 Enz-1(c)D.II

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	David C. Ward, et al.	Examiner: L.E. Crane, Ph.D.
Serial No.:	07/841,910	Group Art Unit: 1803
Filed:	February 26, 1992	
For:	**MODIFIED NUCLEOTIDES AND METHODS OF PREPARING AND USING SAME**	

Lieberman & Nowak
292 Madison Avenue
New York, New York 10017

January 20, 1994

Hon. Commissioner of Patents
and Trademarks
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COMMUNICATION SUBMITTING TERMINAL DISCLAIMER

SIR:

Applicants hereby submit a Terminal Disclaimer in which the assignee of record, Yale University, disclaims the terminal part of any patent granted on the present application that extends beyond the expiration date of U.S. Patent No. 4,711,955. As described in the enclosed Terminal Disclaimer and its attached exhibits, Yale University is the assignee and owner of the subject application and of U.S. Patent No. 4,711,955.

The enclosed Terminal Disclaimer is submitted to overcome the Examiner's rejection, set forth in the May 19, 1993 Office Action, in which all the pending claims were rejected under the judicially created doctrine of obviousness-type double patenting over the claims of U.S. Patent No. 4,711,955. As indicated in Applicants' November 19, 1993 Amendment in response to the Office Action, Applicants do not agree that the claims of the present application are obvious variations of the claims of U.S. Patent No. 4,711,955, but submit the Terminal Disclaimer in order to advance prosecution of the subject application.

No fee is deemed necessary in connection with the filing of this Communication. If any fee is required, authorization is hereby given to charge the amount of any such fee to deposit account no. 12-1325.

Respectfully submitted,

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I hereby declare that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231

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NOTICE OF ALLOWANCE AND ISSUE FEE DUE

☐ Note attached communication from the Examiner
☐ This notice is issued in view of applicant's communication filed _____

SERIES CODE/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
07/532,704	06/04/90	031	ROLLINS, J 1803	09/16/92

First Named Applicant: ENGELHARDT, DEAN

TITLE OF INVENTION: BASE MOIETY-LABELED DETECTABLE NUCLEOTIDE (AS AMENDED)

BUC	ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1	ENZ-5DIV.4	536-027.000	633	UTILITY	NO	$1130.00	12/16/92

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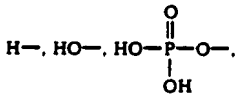
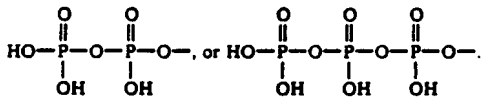
IMPORTANT REMINDER: Patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees.

B is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a purine, A is attached to the 8-position of the purine, if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a detectable complex with one of avidin, streptavidin or antibodies to biotin or iminobiotin; and wherein Z represents:

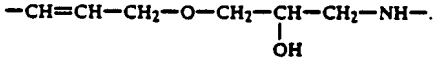




10. The sequence in accordance with claim 9, wherein B is deazaadenine or deazaguanine.

11. The sequence in accordance with claim 9, wherein said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a $—CH_2—NH—$.

12. The sequence in accordance with claim 11, wherein the linkage group is characterized by a moiety selected from the group consisting of —CH=CH—CH_2—NH— and

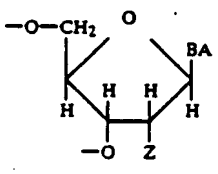


13. The sequence in accordance with claim 9, wherein z is —OH or —H.

14. The sequence in accordance with claim 9, wherein B is a cytosine or uracil.

15. A double-stranded RNA or DNA duplex or DNA-RNA hybrid which comprises:

(i) in one strand a poly- or oligonucleotide sequence which comprises at least one of a moiety having the structure:

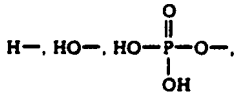


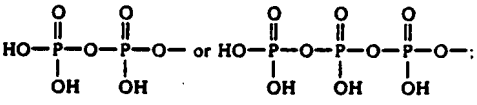
wherein B represents a purine, a 7-deazapurine or a pyrimidine moiety covalently bonded to the C^1-position of the sugar moiety provided that when B is a purine or a 7-deazapurine, it is attached at the N^9-position of the purine or deaazapurine, and when b is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a purine, A is attached to the 8-position of the purine, if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a detectable complex with one of avidin, streptavidin or antibodies to biotin or iminobiotin; and

wherein Z represents





and

(ii) in the second strand, a poly- or oligonucleotide sequence containing neither a biotin nor iminobiotin.

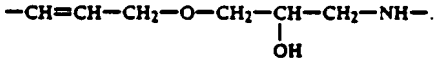
16. The duplex or hybrid in accordance with claim 15, wherein z is H— or HO—.

17. The duplex or hybrid in accordance with claim 15, wherein B is a pyrimidine or a 7-deazapurine.

18. The duplex or hybrid in accordance with claim 17, wherein B is a uracil, cytosine, deazaadenine or deazaguanine.

19. The duplex or hybrid in accordance with claim 15, wherein said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a $—CH_2—NH—$.

20. The duplex or hybrid in accordance with claim 19, wherein the linkage group is characterized by a moiety selected from the group consisting of —CH=CH—CH_2—NH— and



21. A nucleotide or oligo- or polynucleotide sequence comprising at least one of a moiety having the structure:

wherein B represents a purine;

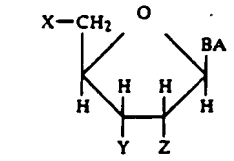
wherein A represents a moiety selected from the group consisting of biotin and iminobiotin, attached to the 8-position of the purine, A being attached to B through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a detectable complex with one of avidin, streptavadin or antibodies to biotin or iminobiotin, and being characterized by an olefinic bond at the α-position relative to B.

* * * * *

bacteria to which a particular fragment of DNA hybridizes the sensitivity must be such that a single labelled structure can be detected. This can be done using a low-light-level video system and computer summation of images, or by using some other device for intensifying the light image. A flow system can also be used if the sensitivity can be made sufficiently grand. If one immobilized the bacteria on a slide their location could be found and the number of such fluorescent spots counted. This would provide a count of all of those bacteria which contain DNA which can hydridize whith the specific clone utilized. If the clone is selected as being specific for a particular strain or bacteria, then one can count the number of organisms of that strain. In addition, any antibiotic resistance for which a particular gene has been identified could be characterized in a similar way using, as a probe, the DNA sequence which is contained in the antibiotic resistance gene. In addition, a probe could be used which is specific for a resistance plasmid containing one or more antibiotic resistance genes. In addition to individual bacteria, groups of bacterial cells of a particular strain can be detected and their number estimated if they are located in a small spot so that the total fluorescence specific to the hybridized DNA in the spot can be measured. In this way the number of organisms containing a specific DNA sequence can be measured in a mixture of bacteria.

What is claimed is:

1. A nucleotide or oligo- or polynucleotide sequence comprising at least one of a moiety having the structure:

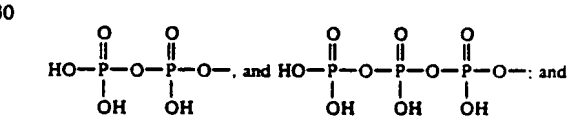


wherein B represents a 7-deazapurine or a pyrimidine moiety covalently bonded to the C$^{1'}$-position of the sugar moiety, provided that when B is a 7-deazapurine, it is attached at the N^9-position of the deazapurine, and when B is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a detectable complex with one of avidin, streptavidin or antibodies to biotin or iminobiotin;

wherein X represents a moiety selected from the group consisting of:

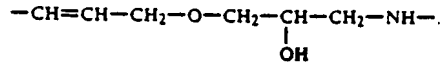


wherein Y is —OH and Z is —OH or —H.

2. The compound in accordance with claim 1, wherein B is a cytosine, uracil, deazaadenine or deazaguanine.

3. The nucleotide, or oligo- or polynucleotide sequence according to claim 1, wherein said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a —CH$_2$NH—.

4. The nucleotide or oligo- or polynucleotide sequence in accordance with claim 3, wherein the linkage group is characterized by the moiety —CH=CH—CH$_2$—NH— or



5. A compound having the structure:

wherein B represents a 7-deazapurine or a pyrimidine moiety covalently bonded to the C$^{1'}$-position of the sugar moiety, provided that when B is a 7-deazapurine, it is attached at the N^9-position of the deazapurine, and when B is a pyrimidine, it is attached at the N^1-position of the pyrimidine;

wherein A represents a moiety selected from the group consisting of biotin and iminobiotin;

provided that if B is a 7-deazapurine, A is attached to the 7-position of the deazapurine, and if B is a pyrimidine, A is attached to the 5-position of the pyrimidine, A being attached to B directly or through a linkage group, said linkage group not interfering substantially with the characteristic ability of A to form a detectable complex with one of avidin, streptavidin or antibodies to biotin or iminobiotin;

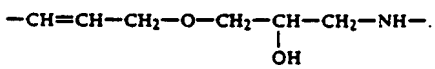
wherein X represents a moiety selected from the group consisting of:

wherein Y is —OH and Z is —OH or —H.

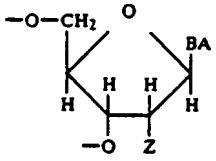
6. The compound in accordance with claim 5, wherein B is a uracil, cytosine, deazaadenine or deazaguanine.

7. The compound in accordance with claim 5, wherein said linkage group is characterized by at least one of an olefinic bond at the α-position relative to B and a —CH$_2$—NH—.

8. The compound in accordance with claim 7, wherein the linkage group is characterized by a moiety selected from the group consisting of —CH=CH—CH$_2$—NH— and



9. A poly- or oligonucleotide sequence which comprises at least one of a moiety having the structure:



wherein B represents a purine, a 7-deazapurine or a pyrimidine moiety covalently bonded to the C$^{1'}$-position of the sugar moiety, provided that when B is a purine or a 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when

iminobiotin was prepared using the protocol previously described for the synthesis of NHS-Biotin (H. Heitzmann and F. M. Richards, Proc. Nat. Acad. Sci. USA, 71, 5537, 1974). AA-UTP (7.0 mg, 0.01 mmole) or AA-dUTP (6.3 mg, 0.01 mmole), prepared as detailed in example 1 (part b), was dissolved in 5 ml of 0.1M sodium borate buffer at pH 8.5, and NHS-iminobiotin (3.5 mg, 0.01 mmole), dissolved in 0.5 ml of dimethylformamide, was added. The reaction mixture was left at room temperature for 12 hours and then loaded directly onto a 10 ml column of DEAE-Sephadex A-25, preequilibrated with 0.05M TEAB at pH 7.5. The column was eluted with a 150 ml linear gradient (0.05–0.6M) of TEAB. Fractions containing iminobiotin-UTP or minobiotin-dUTP, which eluted between 0.35 and 0.40M TEAB, were desalted by rotary evaporation in the presence of methanol and dissolved in H_2O. The products contained a small amount of allylamine-nucleotide adduct as an impurity, as judged by a weak positive result in the ninhydrin test. Final purification was achieved by affinity chromatography on avidin-sepharose. Fractions of the impure product, made 0.1M in soldium borate buffer at pH 8.5, were applied to a 5 ml column of avidin-sepharose and washed with 25 ml of the same buffer. The column was then washed with 50 mM ammonium acetate buffer at pH 4.0, which eluted the desired iminobiotin-nucleotide product in a sharp peak. The nucleotide was precipitated by the addition of 3 volumes of cold ethanol, washed with ethylether, dried in vacuo over sodium hydroxide pellets and stored in a dessicator at −20° C. Products were characterized by elemental analysis, as well as by spectral and chromotographic properties.

EXAMPLES 7 AND 8

Synthesis of NAGE-UTP and NAGE-dUTP

Allyl (3-amino-2-hydroxy-)propyl ether, abbreviated NAGE, was prepared from allyl glycidyl ether (Age) (obtained from Aldrich Chemical Co.). 10 ml of Age (84 mmole) was added slowly (in a fume hood) to 50 ml of 9M ammonium hydroxide and the mixture allowed to stand at room temperature for six hours. Excess ammonia was removed by rotary evaporation under reduced pressure to yield a viscous yellow oil. Analysis of this product by proton NMR showed that it possessed the required structure. 5-mercuri-dUTP (0.1 mmole) or 5-mercuri-UTP (0.2 mmole) was dissolved in 2–4 ml of 0.2M sodium acetate buffer at pH 5.0, and a 16 fold molar excess of NAGE adjusted to pH 5.0 with acetic acid prior to use, was added. The final reaction volumes (4.3 and 8.4 ml) had nucleotide concentrations of 43 and 42 mM, respectively. One equivalent of K_2PdCl_4 (0.1 or 0.2 mmoles) was added to initiate the reaction. After standing at room temperature for 18 hours, the reaction mixtures were filtered through 0.45 μmM membranes the samples diluted five-fold, and chromatographed on columns of DEAE-Sephadex A-25, using linear gradients (0.1–0.6M) of sodium acetate. Fractions containing the desired products, as judged by their UV spectra and characteristic HPLC elution profiles on Partisil ODS-2, were pooled, diluted, and further purified by rechromatography on DEAE-Sephadex using shallow gradients (0.1–0.5M) of ammonium bicarbonate at pH 8.5. Under these conditions the majority of the NAGE-dUTP (or NAGE-UTP) could be cleanly separated from residual impurities. Proton NMR spectra were obtained at this stage of purification after the nucleotides were lyophilized and redissolved in D_2O. For elemental analysis, the products were converted to their sodium salt form. Typical analytical results: Nage-dUTP ($C_{15}H_{22}N_3O_{16}P_3Na_4.2H_2O$), Theory, C, 24.99; H, 3.63; N, 5.83; P, 12.88. Found, C, 25.39; H, 3.71; N, 5.63; P, 12.88.

EXAMPLE 9

Uses of Labeled DNA Sequences

I. Karyotyping

(a) Select from a human gene library some 100 to 200 clones. Label them as described above, and for each clone locate its place or places of hybridization visually or with a low-light-level video system. For those clones which correspond to a unique sequence gene this determines the location of the cloned DNA on a particular human chromosome. Obtain several clones for each chromosome. Each of these labeled clones can be used to identify particular chromosomes. They can also be used in combination to identify each of the 46 chromosomes as being one of the 22 autosomal pairs or the X or the Y. By allowing one set of labeled clones to hybridize to the chromosomes and then adding a fluorescent stain to the label, the set of clones and their locations can be visualized and will flouresce with a particular color. A second set of labeled clones could then be used and reacted with a second fluorescent dye. The same process can be repeated a number of times. Thus one can, if desired, have several sets of fluorescent labels attached to the cellular DNA at different but specific locations on each of the chromosomes. These labels could be used for visual or computerized automatic karyotyping.

(b) For automatic karyotyping, one could use one set of clones to identify the approximate location of each of the 46 chromosomes by finding sets of spots corresponding to the number of labeling sites on each chromosome. Thus, it is possible by computer analysis of the digitized images to determine if the chromosomes are suitably spread for further analysis. If they are suitably spread, then one can use computer analysis to identify each of the individual chromosomes by the location and distribution of the labelled spots on each one.

By using the fact that the fluorescent spots can be placed at specific locations on each chromosome, one can carry out either manual or automatic karyotyping very much more effectively than without such labels.

II. Diagnosis of Genetic Disorders

By selecting the clones which bind specifically to a particular chromosome, such as number 23, it is possible to count the number of copies of the particular chromosome in a cell even if the chromosomes are not condensed at metaphase. Thus when fetal cells are obtained for prenatal diagnosis of trisomy 21, the diagnosis can be done even if the chromosomes are not condensed at metaphase. If necessary, two sets of labels can be used—one which would be specific for chromosome 23 and one for some other chromosome. By measuring in each cell the ratio of the two labels, which might be of different colors, it is possible to identify the cells which show an abnormal number of chromosomes number 23. This procedure could be used either on slides with a low-light-level video system or in a flow cytometer system using laser excitation. It can be used to determine any abnormal chromosome number.

III. Microoganism Detection and Identification

The labeling of specific sequences of DNA as described above permits identification and counting of individual bacteria. In order to identify the individual

4.11; N, 5.39; P, 13.54] spectrally and chromatographically.

(c) Biotination of AA-dUTP or AA-UTP

Biotinyl-N-hydroxysuccinimide ester (NHSB) was prepared from biotin (Sigma) as described previously (H. Heitzmann and F. M. Richards, proc. Natl. Acad. Sci. USA. 71, 3537 [1974]). AA-dUTP.H_2O (63 mg, 0.1 mmole) or AA-UTP.$4H_2O$ (70 mg, 0.1 mmole) was dissolved in 20 ml of 0.1M sodium borate buffer at pH 8.5, and NHSB (34.1 mg, 0.1 mmole) dissolved in 2 ml of dimethyl formamide, was added. The reaction mixture was left at room temperature for four hours and then loaded directly onto a 30 ml column of DEAE-Sephadex TM A-25, preequilibrated with 0.1M TEAB at pH 7.5. The column was eluted with a 400 ml linear gradient (0.1–0.9M) of TEAB. Fractions containing biotinyl-dUTP or biotinyl-UTP, which eluted between 0.55 and 0.65M TEAB, were desalted by rotary evaporation in the presence of methanol and redissolved in water. Occaionally a slightly cloudy solution was obtained: this turbidity, due to a contaminant in some TEAB solutions, was removed by filtration through a 0.45 mm filter. For long term storage, the nucleotides were converted to the sodium salt by briefly stirring the solution in the presence of Dowex TM 50 (Na^+ form). After filtration the nucleotide was precipitated by the addition of three volumes of cold ethanol, washed with ethyl ether, dried in vacuo over sodium hydroxide pellets, and stored in a dessicator at −20° C. For immediate use, the nucleotide solution was made 20 mM in Tris-HCl at pH 7.5, and adjusted to a final nucleotide concentration of 5 mM. Stock solutions were stored frozen at −20° C.

Elemental analysis of the bio-dUTP and bio-UTP products yielded the following results. Bio-dUTP ($C_{22}H_{30}N_5O_{18}P_3S_1Na_4.1H_2O$). Theoretical; C, 29.80; H, 3.38; N, 7.89; P, 10.47; S. 3.61. Found; C, 30.14 H, 3.22; N, 7.63; P, 10.31; S, 3.70. Bio-UTP ($C_{22}H_{30}N_5O_{19}P_3S_1Na_4.3H_2O$): Theoretical; C, 29.15; H, 3.19; N, 7.45; P, 9.89; S, 3.41. Found; C, 28.76; H, 3.35; N, 7.68; P, 9.81; S, 3.32.

The spectral properties of bio-dUTP and bio-UTP at pH 7.5 [λmax, 289 nm (ε=7,100), λmax, 240 nm (ε=10,700); λmin, 262 nm (ε=4,300)] reflect the presence of an exocylic double-bond in conjugation with the pyrimidine ring. These nucleotides also give a strong positive reaction (an orange-red color) when treated with p-dimethylaminocinnamaldehyde in ethanolic sulfuric acid, a procedure used for biotin quantitation (D. B. McCormick and J. A. Roth, Anal. Biochem., 34, 326, 1970). However, they no longer react with ninhydrin, a characteristic reaction of the AA-dUTP and AA-UTP starting materials.

EXAMPLES 3 AND 4

Synthesis of biotinyl-CTP and biotinyl-dCTP

CTP and dCTP were (a) mercurated, (b) reacted with allylamine, and (c) biotinized with NHS-biotin, essentially as described in Example 1. CTP (56.3 mg, 0.1 mmole) or dCTP (59.1 mg, 0.1 mmole) were dissolved in 20 ml of 0.1M sodium acetate buffer at pH 5.0, and mercuric acetate (0.159 gm, 0.5 mmoles) added. The solution was heated at 50° C. for 4.5 hours then cooled on ice. Lithium chloride (39.2 mg, 0.9 mmoles) was added and the solution extracted 6 times with ethyl acetate. The nucleotide products in the aqueous layer were precipitated by the addition of three volumes of cold ethanol and the precipitate collected by centrifugation. The precipitate was washed with absolute ethanol, ethyl ether, and then air dried. These products were used without further purification for the synthesis of AA-CTP and AA-dCTP, respectively. The mercurated nucleotides were dissolved in 0.1M sodium acetate buffer at pH 5.0 and adjusted to a concentration of 10 mM (92 OD/ml at 275 nm). 0.6 ml (1.2 mmole) of a 2.0M allylamine acetate stock (prepared as described in Example 1) was added to 10 ml of nucleotide solution (0.1 mmole) followed by the addition of K_2PdCl_4 (32.6 mg, 0.1 mmole), dissolved in 1.0 ml of H_2O. After standing at room temperature for 24 hours, the solution was filtered through a 0.45 mM membrane to remove metal precipates. The filtrate was diluted five-fold and loaded onto a 50 ml column of DEAE-sephadex A-25, preequilibrated with 50 mM TEAB at pH 7.5. The nucleotide products were fractionated by application of a 500 ml linear gradient (0.05–0.6M) of TEAB at pH 7.5. The desired product was in the major UV absorbing portion which eluted between 0.28 and 0.38M salt. The pooled samples were desalted by rotary evaporation, dissolved in 0.5M triethylammonium acetate at pH 4.2, and final purification achieved by HPLC chromatography on columns of Partisil ODS-2, using 0.5M triethylammonium acetate as the eluent. Appropriate fractions were pooled, lyophilized, and the products dissolved in H_2O. The nucleotides were converted to the Na^+ salt by stirring briefly in the presence of Dowex TM 50 (Na^+ form). After filtration, to remove the Dowex resin, the nucleotides were precipitated by the addition of 3 volumes of cold ethanol. The precipitate was washed with ether and then air dried. Analytical results: AA-dCTP ($C_{12}H_{17}N_4O_{13}P_3Na_4.2H_2$); Theory, C, 22.29; H, 2.63; N, 8.67, P, 14.40. Found C, 22.16; H. 2.89; N. 8.77; P, 14.18. AA-CTP ($C_{12}H_{17}N_4O_{14}Na_4.2H_2O$); Theory C, 21.75; H, 2.57; N, 8.46; P, 14.01. Found, C, 22.03; H, 2.47; N, 8.69; P, 13.81; Spectral properties in 0.1M Borate buffer at pH 8.0, λmax 301 nm (ε=6,400), λmin 271 nm (ε=3,950) λmax 250 nm (ε=9.700). Both AA-dCTP and AA-CTP give a positive ninhydrin test. AA-CTP (6.6 mg, 0.01 mmole) or AA-dCTP (6.4 mg, 0.01 mmole) was dissolved in 5 ml of 0.1M sodium borate buffer at pH 8.5, and NHS-biotin (3.4 mg, 0.01 mmole), dissolved in 0.2 ml of dimethylformamide, was added. After sitting at room temperature for 4 hours the sample was chromatographed on a 10 ml column of DEAE-Sephadex A-25, using a 150 ml linear gradient (0.1–0.9M) of TEAB at pH 7.5, as eluent. Fractions containing biotinyl-CTP or biotinyl-dCTP, which eluted between 0.50 and 0.60M TEAB, were pooled, desalted by rotary evaporation, and after being adjusted to a final concentration of 5 mM in 0.02M Tris-HCl buffer at pH 7.5, were frozen at −20° C. The products give a strong positive reaction for biotin with p-dimethylaminocinnamldehyde in ethanolic sulfuric acid but give a negative test for primary amines when sprayed with ninhydrin. Further structural characterization of these products is in progress.

EXAMPLES 5 AND 6

Synthesis of Iminobiotinyl-UTP and Iminobiotinyl-dUTP

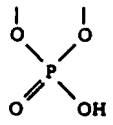
Iminobiotin hydrobromide was prepared from biotin as described previously (K. Hofmann, D. B. Melville and V. du Vigneaud, J. Biol. Chem., 141, 207–211, 1941; K. Hofmann and A. E. Axelrod, Ibid., 187, 29–33, 1950). The N-hydroxysuccinimide (NHS) ester of

which each point, i.e. each pixel, of the image is strictly proportional to the number of photons emitted by a point at the object. Using systems of this kind or flow systems in which the cells or parts of cells flow past a laser beam, one can obtain detection sensitivity increases for fluorescent material of factors between 100 and 1000 beyond that which can be detected by the eye. This increase is sufficient to detect the fluorescence of single copy genes.

In a preferred modification, analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm have been synthesized. These biotinylnucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro. DNA containing low levels of biotin substitution (50 molecules or less/kilobase) has denaturation, reassociation and hybridization characteristics which are indistinguishable from that of unsubstituted control DNA.

Thus, this invention also provides a method of chromosomal karyotyping. In this method, modified polynucleotides are paepared which correspond to known genes and include modfied nucleotides. These polynucleotides are hybridized with chromosomal deoxyribonucleic acid and the resulting duplexes contacted with appropriate polypeptides under suitable conditions to permit complex formation. The polypeptides include detectable moieties so that the location of the complexes can be determined and the location of specific genes thereby fixed.

Another embodiment of this invention involves detection of poly A-containing sequences using poly U in which some of the uracil bases have been modified to contain a probe. Yet another embodiment involves cyclic modified nucleotides in which two of x, y and z are reacted to form the cyclic moiety



Such cyclic modified nucleotides may then be used to identify hormone receptor sites on cell surfaces which in turn can be used as a method of detecting cancer or tumor cells.

Finally, tumor cells can be diagnosed by preparing polynucleotides which are modified according to this invention and are complementary to the messenger ribonucleic acid synthesized from a deoxyribonucleic acid gene sequence associated with the production of polypeptides, such as α-fetal protein or carcinoembryonic antigen, the presence of which is diagnostic for specific tumor cells. Hybridization and detection of hybrid duplexes thus would provide a method for detecting the tumor cells.

The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way its scope as more particularly set forth in the claims.

EXAMPLE 1 AND 2

Synthesis of biotinyl-UTP and biotinyl-dUTP

(a) Preparation of Mercurated Nucleotides

UTP (570 mg, 1.0 mmole) or dUTP 554 mg, 1.0 mmole) was dissolved in 100 ml of 0.1M sodium acetate buffer pH 6.0, and mercuric acetate (1.59 gm, b 5.0 mmoles) added. The solution was heated at 50° C. for 4 hours, then cooled on ice. Lithium chloride (392 mg, 9.0 mmoles) was added and the solution extracted six times with an equal volume of ethyl acetate to remove excess $HgCl_2$. The efficiency of the extraction process was monitored by estimating the mercuric ion concentration in the organic layer using 4,4'-bis(dimethylamino)-thiobenzophenone (A. N. Christoper, Analysis, 94, 392 (1969). The extent of nucleotide mercuration, determined spectrophotometrically following iodination of an aliquot of the aqueous solution as described by Dale et al. (R. M. K. Dale, D. C. Ward, D. C. Livingston, and E. Martin, Nucleic Acid Res. 2, 915 [1975], was routinely between 90 and 100%. The nucleotide products in the aqueous layer, which often became cloudy during the ethyl acetate extraction, were precipitated by the addition of three volumes of ice-cold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol, once with ethyl ether, and then air dried. These thus prepared mercurated nucleotides were used for the synthesis of the allylamine-nucleotides without further purification.

(b) Synthesis of allylamine-dUTP and allylamine-UTP

The mercurated nucleotides (of step a) were dissolved in 0.1M sodium acetate buffer at pH 5.0, and adjusted to a concentration of 20 mM (200 OD/ml at 267 nm). A fresh 2.0M solution of allylamine acetate in aqueous acetic acid was prepared by slowly adding 1.5 ml of allylamine (13.3 mmoles) to 8.5 ml of ice-cold 4M acetic acid. Three ml (6.0 mmoles) of the neutralized allylamine stock was added to 25 ml (0.5 mmole) of nucleotide solution. One nucleotide equivalent of K_2PdCl_4, (163 mg, 0.5 mmole), dissolved in 4 ml of water, was then added to initiate the reaction. Upon addition of the palladium salt (Alfa-Ventron) the solution gradually turned black with metal (Hg and Pd) deposits appearing on the walls of the reaction vessel. After standing at room temperature for 18–24 hours, the rection mixture was passed through a 0.45 mm membrane filter (nalgene) to remove most of the remaining metal precipitate. The yellow filtrate was diluted fivefold and applied to a 100 ml column of DEAE-Sephadex TM A-25 (Pharmacia). After washing with one column volume of 0.1M sodium acetate buffer at pH 5.0, the products were eluted using a one liter linear gradient (0.1–0.6M) of either sodium acetate at pH ~ 8–9, or triethylammonium bicarbonate (TEAB) at pH 7.5. The desired product was in the major UV-absorbing portion which eluted between 0.30 and 0.35M salt. Spectral analysis showed that this peak contained several products, final purification was achieved by reverse phase-HPLC chromatography on columns of Partisil-ODS2, using either 0.5M $NH_4H_2PO_4$ buffer at pH 3.3 (analytical separations), or 0.5M triethylammonium acetate at pH 4.3 (preparative separations) as eluents. The 5'-triphosphates of 5-(3-aminopropen-1-yl)uridine (the allylamine adduct to uridine) were the last portions to be eluted from the HPLC column and they were clearly resolved from three, as yet uncharacterized, contaminants. These nucleotides were characterized by proton NMR elemental analysis [AA-dUTP ($C_{12}H_{16}N_3O_{14}P_3Na_4.1H_2O$): theory C, 22.91; H, 2.88; N, 6.68; P, 14.77. Found, C, 23.10; H, 2.85; N, 6.49; P, 14.75. AA-UTP ($C_{12}H_{16}N_3O_{15}P_3Na_4.4H_2O$): Theory, C 20.61; H, 3.46; N, 6.01; P, 13.3. Found C, 20.67; H,

the chromosome and make clinical cytogenetic diagnosis much more rapid and practical.

While a single-step "antibody sandwich" method in which the chromosome spread is challenged, post-hybridization, with rabbit anti-biotin IgG may succeed, this protocol may not generate sufficient fluorescence for unambiguous gene assignments. However, a much stronger fluorometric signal can be achieved by using the "haptene-antibody sandwich technique" described by Lamm, et al, (1972); Wofsy, et al., (1974). In this procedure the primary antibody, in our case monospecific, rabbit anti-biotin IgG, is chemically modified with a haptenization reagent, such as 2,4-dinitrofluorobenzene, preferably while the immunoglobulin is bound to an antigen affinity column (biotin-Sepharose TM). As many as 15–20 haptene (DNP) groups can be coupled to the primary antibody without decreasing its antigen binding affinity or specificity (Wallace and Wofsy, 1979). If the primary antibody treatment of the test sample is followed by an incubation with a fluorescently labeled anti-hapten IgG antibody, rather than a fluorescently labeled anti-IgG, a 5–7 fold increase in fluorescence signal can be achieved. Since one also has available monospecific guinea pig anti-DNP IgG, we can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, DNP-labeled anti-biotin IgG and biotin-labeled anti-DNP IgG. If these can be used alternately to achieve several rounds of hapten-antibody sandwiching and then followed with fluorescently labeled protein A from *Staphylococcus aureus*, which binds specifically to IgG molecules from many mammalian species, it could result in an enormous amplification of the primary antibody signal with its concomitant utility.

The protein streptavidin from *Streptomyces avidini* is a potential alternative to anti-biotin IgG as a vehicle to specifically direct a coupled visualization system [e.g., fluorescent probes (above) or histochemical reagents (below)] to the site of the hybridized biotin-containing polynucleotide. One of streptavidin's advantages over anti-biotin IgG is that its affinity for biotin is $K_{assn}=10^{15}$ whereas association constants for haptene-IgG interactions are 10^7 to 10^{10}. The fast reaction rate and extreme affinity mean that the time required to localize the biotinized probe will be minutes with streptavidin versus hours with immunologic reagents.

Initial evaluations of a streptavidin detection system are currently in progress. Polytene chromosomes hybridized with biotinized DNA probes will be incubated with streptavidin followed by a subsequent incubation with bovine serum albumin which has been doubly labeled with biotin and FITC (FITC, biotinyl-BSA). Since only one of the four streptavidin subunits is likely to be involved in binding at each biotinized DNA site, potentially one labeled BSA molecule can bind to each of the remaining three nonconjugated subunits of the streptavidin-biotinyl nucleotide complex. The fluorescence signal from this single streptavidin+FITC, biotinylBSA layer will be compared with a control using the basic "antibody sandwich method" described earlier.

If the "antibody sandwich" and streptavidin+FITC, biotinyl-BSA detection intensities are comparable, one can attempt to enhance the streptavidin+FITC, biotinyl-BSA system to single-copy copy sensitivity in a manner that parallels the multiple "haptene-antibody sandwich" approach. Since some of biotin groups on BSA will not be bound to the first layer of streptavidin, a second layer of streptavidin can be added until sufficient signal is obtained. For example, if in the second layer, only two streptavidin protomers bind to each first-layer BSA and each of these streptavidin protomers binds three FITC-biotinyl BSA molecules, then the second layer intensity will be twice as great as that from the first layer; for the third layer, with analogous binding stoichiometries, the fluorescent intensity will be 12-fold that of the first layer, so the total intensity will rapidly increase with successively added layers. There are plans to use a larger carrier protein such as thyroglobulin rather than BSA in order to maximize amounts of attached fluorescent and biotin probes. It may also be necessary to use a longer linker arm between the biotin probe and the carrier protein. A longer linker arm should sterically optimize the theoretical delivery of a biotinized fluorescent carrier molecule to each nonconjugated streptavidin subunit and maximize the number of streptavidin protomers in the subsequent layer which will bind to the biotinized fluorescent carrier. As before, appropriate controls will be done to insure that substitution of the carrier protein with fluorescent probes and biotin does not cause solubility and/or nonspecific binding problems.

The streptavidin-carrier protein delivery system has two significant advantages over the immunfluorescent approach in addition to its speed of delivery. First, only two protein components are needed to form the layers. Second, only the carrier protein needs to be modified and it is not necessary to maintain functional or even total structural integrity as long as the biotin groups are accessible to streptavidin.

An alternative to the fluorescence method for visualizing hybridized probes is to direct enzymes such as peroxidase, alkaline phosphatase of β-galactosidase to the hybridization site where enzymatic conversion of soluble substrates to insoluble colored precipitates permits light microscope visualization. The important advantage of this technique is that the histochemical methods are 10 to 100-fold more sensitive than fluorescence detection. In addition, the colored precipitates do not bleach with extensive light exposure thus avoiding one of the general disadvantages of fluorescent light microscopy. These enzymes can be coupled to the final antibody instead of fluorescent probes in the "haptene-antibody sandwich" technique using bifunctional reagents such as glutaraldehyde or in the case of peroxidase via oxidation of the peroxidase carbohydrate moieties to aldehydes and coupling of these residues with ϵ-amino groups of the desired protein. For the streptavidin-biotinized carrier protein method, an enzyme with biotinyl groups coupled to it could replace a fluorescently-biotinized carrier system. Alternately, the enzyme could be coupled via biotin to the last layer of streptavidin with amplification of streptavidin sites being built up in preceding layers using biotinized BSA or thyroglobulin. We will begin developing the necessary histochemical reagents and the appropriate substrate/insoluble product combinations for visualizing in situ hybridizations without background problems in the near future. The histochemical approaches to signal amplification should therefore be ready for trial in the summer of 1981.

Detecting and/or imaging very low levels of fluorescent light is possible using currently available image intensifiers or systems composed of lasers and photomultipliers. These methods permit the detection of light down to the level of individual photons. With suitable digital processing systems, images can be produced in

nosa, Streptococcus pyogenes or *Neisseria gonorrhoeae;* and aminoglycoside resistance in *Mycobacterium tuberculosis* can be determined.

In these methods a polynucleotide is prepared which is complementary to the nucleic acid sequence which characterizes the organism or its antibiotic resistance and which additionally includes one or more modified nucleotides according to this invention. This polynucleotide is hybridized with nucleic acid obtained from the organism under scruntiny. Failure to hybridize indicates absence of the organism or of the resistance characteristic. Hybridized nucleic acid duplexes are then identified by forming a complex between the duplex and a suitable polypeptide which carries a detectable moiety, and detecting the presence of the complex using an appropriate detection technique. Positive detection indicates that the complex, the duplex and therefore the nucleic acid sesequence of interest are present.

This approach can be extended to the diagnosis of genetic disorders, such as thalassemia and sickle cell anemia. The deoxyribonucleotide acid gene sequence whose presence or absence (in the case of thalassemia) is associated with the disorder can be detected following hybridization with a polynucleotide probe according to this invention based upon complex formation with a suitable detectable polypeptide.

The mapping of genes or their transcripts to specific loci or chromosomes has been a tedious and time-consuming occupation, involving mainly techniques of cellfusion and somatic cell genetics. Although in situ hybridization has been employed successfully for mapping single-copy gene sequences in species that undergo chromosomes polytenization, such as Drosophila, detection of unique sequence genes in most higher eukaryotic chromosomes has been extremely difficult, if not impossible, using standard hybrization methods. The necessity for polynucleotide probes of very high specific radioactivity to facilitate autoradiographic localization of the hybridization site also results in rapid radiodecomposition of the probe and a concomitant increase in the background noise of silver grain deposition. The use of hybridization probes with low to moderate specific radioactivities requires exposure times of many days or weeks, even to detect multicopy sequences, such as ribosomal RNA genes or satellite DNA. Since recombinant DNA technology has made feasible the molecular cloning of virtually every single-copy sequence found in eukaryotic cells, it would be extremely beneficial to have a rapid and sensitive method for mapping the chromosomal origin of such cloned genomic fragments.

Modified nucleotides may be used in a method of gene mapping by in situ hybridization which circumvents the use of radioisotopes. This procedure takes advantage of a thymidine analogue containing biotin that can be incorporated enzymatically into DNA probes by nick translation. After hybridization in situ the biotin molecules serve as antigens for affinity purified rabbit anti-biotin antibodies. Immunofluorescent antibody sandwiches made with fluorescein-labeled goat anti-rabbit IgG allow for rapid and specific cytogenetic localization of cloned gene sequences as green-yellow bands. This method offers four major advantages over conventional autoradiographic methods of in situ gene localization; less background noise, an increase in resolving power between bands; a decrease in the time required to determine the site of probe hybridization; and chemically stable hybridization probes. This method has been applied successfully to the localization of reitterated and unique DNA sequences in the polytene chromosome of *Drosophila milanogaster* and satellite DNA on mouse metaphase chromosomes.

Thus it has been found that polytene chromosomes could be used as a test system for establishing the efficacy of probes using the modified nucleotides according to the instant invention as detected by indirect immunofluorescence for in situ gene mapping. The probes included a variety of cloned Drosophila sequences obtained from Otto Schmidt and Dieter Söll, such as tRNA genes cloned in plasmid vectors with inserts of sizes ranging from about 5 to about 22 kilobases. Many of these clones have already been assigned to specific bands on the Drosophila chromosome map by conventional in situ hybridization methods employing radioisotopes.

DNA probes were nick translated in the presence of Bio-dUTP. Occasionally ^{3}H dATP and/or ^{3}H dCTP was included in the nick translation reaction mixture. This allowed both autoradiographic and immunofluorescent localization of a sequence on a single chromosone spread. In situ hybridization was performed as described in M. L. Pardue, and J. G. Gall, Methods in Cell Biol., 10, 1 (1975). After the final 2×SSC wash to remove unhybridized probe, the slides were rinsed with PBS (phosphate buffered saline) and incubated at 37° C. with 2.5 μg/ml Rabbit anti-biotin in PBS and 10 mg/ml BSA for 2-16 hours. This was followed by incubation of the slides with FITC labeled Goat anti-Rabbit IgG (Miles Laboratories, diluted 1:100 in PBS and 10 mg/ml BSA) for one-four hours. Evans Blue was often required as a red counterstain to see the chromosomes with fluorescent illumination.

When plasmids pBR 17D and pPW 539 containing 5 Kb and 22 Kb inserts, respectively, were hybridized by this method, it was found that the pattern of hybridization is reproducible from spread to spread and is observed unambiguously on greater than 90% of the chromosome spreads on a given slide.

The cloned transposable element pAC 104 is known to map at many sites along the Drosophila genome. Comparison of the autoradiograph and the fluorescent picture obtained by in situ hybridization of this probe illustrates a major advantage of this method, i.e., that where diffuse regions of silver grains appear on an autoradiograph, doublets or a series of bands are discernible by immunofluorescent labeling.

The other immediately obvious advantage of this method is the tremendous decrease in time required for gene assignments to be made by indirect immunofluorescence. An assignment of a DNA fragment to a specific band can be made within six hours of hybridization. This is in comparison to days or weeks required for autoradiographic exposure methods. This factor, in combination with increased resolution, makes the use of modified nucleotides detected by indirect immunofluorescence immediately preferable to more classical methods.

It has been shown that this immunological method also works with mammalian chromosomes wherein satellite DNA has been mapped to the centromeric regions of mouse metaphase chromosomes. The result provides a basic foundation for the development of a simple gene mapping procedure for single copy (unique) sequences in chromosomes from human and other mammals. Such a procedure should greatly facilitate our understanding of the genetic organization of

The modified polynucleotides of this invention are capable of denaturation and renaturation under conditions compatible with their use as hybridization probes. An analysis of the thermal denaturation profiles and hybridization properties of several biotin-substituted DNA and RNA polymers clearly indicates this. For example, pBR 322 DNA or λDNA, nick translated to introduce approximately 10–100 biotin residues per kilobase, have Tm values essentially identical to that of the control, biotin-free DNAs. Furthermore, ^{32}P-labeled, biotin-substituted, pBR 322 DNA, exhibited the same degree of specificity and autoradiographic signal intensity as control, thymidine-containing DNA, when used as a hybridization probe for detecting bacterial colonies containing the plasmid.

In DNA duplexes, such as MVM RF DNA, in which every thymidine residue in one strand (1250 in toto per 5 Kb) is replaced by a biotinyl-nucleotide, the Tm is only 5° C. less than that of the unsubstituted control. Although the Tm of poly d(A-bioU) in which each base pair contains a bio-dUMP residue is 15° C. lower than the poly d(A-T) control, the degree of cooperativity and the extent of hyperchromicity observed both during denaturation and renaturation were the same for the two polymers. A parallel analysis of RNA duplexes and DNA/RNA hybrids indicates that their Tm's also decrease as the biotin-content of the polymer increases. However, it is clear that a substantial number of biotin-molecules can be introduced without significantly altering the hybridization characteristics of the polymers.

These results strongly suggested that biotin-substituted polynucleotides could be used as probes for detecting and/or localizing specific polynucleotide sequences in chromosomes, fixed cells, or tissue sections. The general protocol for detecting the biotin-substituted probe is schematically illustrated as follows:

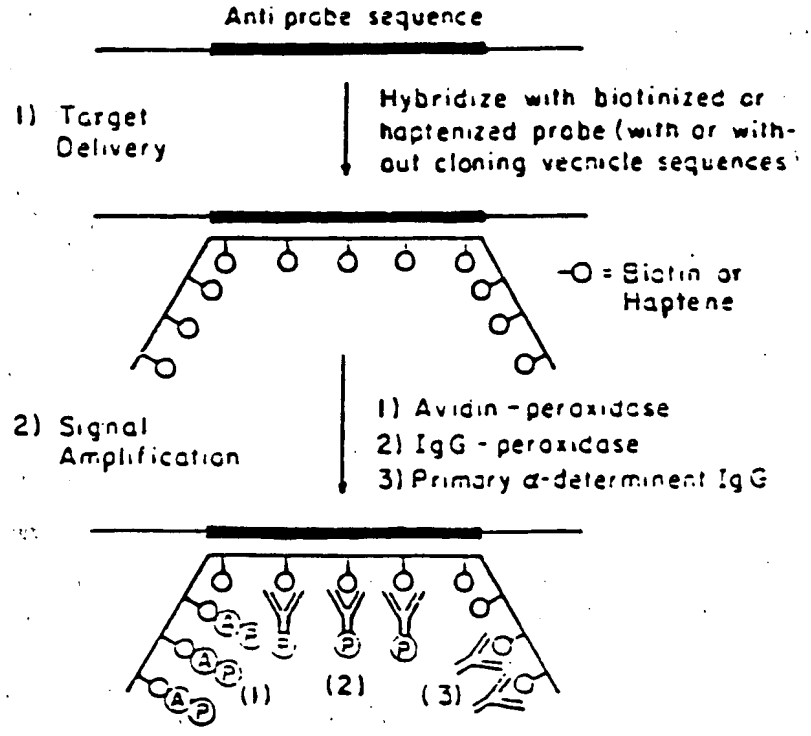
This general scheme illustrates only procedures used for gene mapping (cytogenetics), and recombinant DNA-technologies. However, it can be equally well applied to the detection of nucleic acid sequences of bacterial, viral, fungal or parasite origin in clinical samples and this forms the basis of a powerful new approach to clinical diagnostics which does not rely on the use of radioisotopes.

Immunological and histochemical methods for the detection of biotin have shown that the basic approach is useable for a rapid method of gene mapping in situ hybridization and non-radioactive procedures for detecting specific nucleic acid sequences by blotting hybridization methods. Use may be made of this technology in development of new clinical diagnostic procedures.

Using this approach, it is possible to determine the presence of a specific deoxyribonucleic or ribonucleic acid molecule, particularly such a molecule derived from a living organism, e.g. bacteria, fungus, virus, yeast, or mammal. This in turn permits diagnosis of nucleic acid-containing etiological agents in a patient or other subject.

Moreover, it provides a method for screening bacteria to determine antibiotic resistance. Thus, for example, penicillin resistance in *Streptococcus pyogenes* or *Neisseris meningitidis;* tetracycline resistance in *Staphylococcus aureus, Candida albicans, Pseudomonas aerugi-*

GENERAL PROTOCOL FOR PROBE DETECTION VIA *IN SITU*, COLONY OR NORTHERN/SOUTHERN HYBRIDIZATION METHODS



wherein each of B, B', and B" represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that whenever B, B', or B" is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and whenever B, B', or B" is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded duplex formed with a complementary ribonucleic or deoxyribonucleic acid molecule.

wherein the dotted line represents a linkage group joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine;

wherein z represents H— or HO—; and

wherein m and n represent integers from 0 up to about 100,000.

Of course, it should be readily understood that in general m and n will not simultaneously be 0 since, in that event, the compound becomes merely a modified nucleotide as described previously. In general B' and B" will vary within the same oligo- or polynucleotide, being alternatively uracil, cytosine, thymine, guanine, adenine, or the like. Also, in general, the variation will correspond to the ordered sequence of nucleotides which codes for the synthesis of peptides according to the well known Genetic Code. However, it is intended that the structure shown also embrace polynucleotides such as poly C, poly U, poly r(A-U), and poly d(A-U) as well as calf thymus DNA, ribosomal RNA of *E. coli* or yeast, bacteriophage RNA and DNA (R17, fd), animal viruses (SV40 DNA), chromosomal DNA, and the like, provided only that the polynucleotides be modified in accordance with this invention.

It is also to be understood that the structure embraces more than one modified nucleotide present in the oligomer or polymer, for example, from two to thirty modified nucleotides. The critical factor in this regard is that the number of modifications not be so great that the polynucleotide is rendered ineffective for the intended use.

Finally, it should be understood that modified oligo- and polynucleotides can be joined to form larger entities having the same structure so long as terminal groups are rendered compatible or reactive.

These compounds can be made by enzymatic polymerization of appropriate nucleotides, especially nucleotide triphosphates in the presence of a nucleic acid template which directs synthesis under suitable conditions. Such conditions can vary widely depending upon the enzyme employed, amounts of nucleotides present, and other variables. Illustrative enzymes include DNA polymerase I of *E. coli*, bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine and human (HeLa) cells, DNA polymerase from Herpes simplex virus, RNA polymerase of *E. coli*, RNA polymerase of bacteriophage T7, eukarotic RNA polymerase including HeLa cell RNA polymerase III, calf thymus RNA polymerase II, and mouse cell RNA polymerase II.

Also, the compounds can be prepared by terminal addition to oligo- or polynucleotides to produce compounds in which m or n is 0 depending upon whether the addition is at the 5' or 3' position. Moreover, the compounds such as pCp or pUp in which the base is biotinized can be added to existing molecules employing the enzyme RNA ligase.

Modified oligo- and polynucleotides can also be prepared by chemical modification of existing oligo- or polynucleotides using the approach described previously for modification of individual nucleotides.

The various modified nucleotides, oligonucleotides, and polynucleotides of this invention may be detected by contacting the compounds with polypeptides which are capable of forming complexes therewith under suitable conditions so as to form the complexes, provided that the polypeptides include one or more moieties which can be detected when the complex or complexes is or are formed, generally by means of conventional detection techniques.

One polypeptide detector for the biotinyl-type probe is avidin. The avidin-biotin interaction exhibits one of the tightest non-covalent binding constants ($K_{dis}=10^{-15}$) seen in nature. If avidin is coupled to potentially demonstrable indicator molecules, e.g., fluorescent dyes (fluoroscein, rhodamine), electron-dense reagents (ferritin, hemocyanin, colloidal gold), or enzymes capable of depositing insoluble reaction products (peroxidase, alkaline phosphatase) the presence, location and/or quantity of the biotin probe can be established.

Avidin has, unfortunately, one property that makes it less desirable as a biotin-indicator protein when used in conjunction with nucleic acids or chromatin material. It has been reported (M. H. Heggeness, Stain Technol., 52, 165, 1977; M. H. Heggeness and J. F. Ash, J. Cell Biol., 73, 783, 1977; E. A. Bayer and M. Wilchek, Methods of Biochemical Analysis 26, 1, 1980) that avidin binds tightly to condensed chromatin or to subcellular fractions that contain large amounts of nucleic acid in a manner which is independent of its biotin-binding property. Since avidin is a basic glycoprotein with a pI of 10.5, its histone-like character or its carbohydrate moieties are most likely responsible for these observed non-specific interactions.

A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein synthesized by the soil organism *Streptomyces avidinii*. Its preparation and purification is described in Hoffman, et al., Proc. Natl. Acad. Sci., 77, 4666 (1980). Streptavidin has a much lower pI (5.0), is non-glycosylated, and shows much lower non-specific binding to DNA than avidin, and therefore offers potential advantages in applications involving nucleic acid detection methodology.

A most preferred protein for biotin-like probe detection is monospecific rabbit IgG, antibiotin immunoglobulin. This compound was prepared by immunizing rabbits with bovine serum albumin conjugated biotin as described previously (M. Berger, Methods in Enzymology, 62, 319 [1979]) and purified by affinity chromatography. Although the association constant of immunoglobulin-haptens have values of K_{assn} (10^6 to 10^{10}) which are considerably lower than for avidin-biotin complexes, they are substantially equivalent to those observed with the avidin-iminobiotin complex. Furthermore, the anti-biotin antibodies have proven extremely useful in detecting specific polynucleotide sequences on chromosomes by in situ hybridization since little, if any, non-specific binding of the antibody to chromatin material occurs.

clearly show that the biotinyl-nucleotides were incorporated. The first is that polynucleotides synthesized in the presence of biotin-nucleotides are selectively retained when chromatographed over avidin or streptavidin affinity columns. (Tables I and II) For example, whereas normal DNA, nick translated with ^{32}P-dAMP, is quantitatively eluted upon the addition of 0.5M NaCl, the vast majority of biotinyl-DNA or iminobiotinyl-DNA remains bound to the resin even after extensive washing with high salt, urea, quanidine-HCl, formamide or 50 mM NaOH. The small fraction of the radiolabel eluted by these washing conditions is not retained when applied to the resin a second time, suggesting that radioactivity is associated with DNA fragments which are free of biotin substitution. The second line of evidence is that only biotin-labeled polynucleotides are immunoprecipitated when treated with purified anti-biotin IgG followed by formalin-fixed *Staphylococcus aureus*. (Table III) It is clear from the data in these tables that extremely small amounts of biotin can be detected by this method. These results also show that the biotin molecule can be recognized by avidin, streptavidin or specific antibodies while the DNA is still in its native, double-stranded form, a condition that is absolutely essential if the antibody-binding or avidin-affinity approaches are to be useful in probe detection employing hybridization techniques.

TABLE I

SELECTIVE RETENTION OF BIOTINIZED DNA ON AVIDIN-SEPHAROSE

Eluent	% DNA Retained on Resin	
	Bio-DNA (1%)	T-DNA
Load - 3 × 10^5 cpm 10 mM Tris 7.5 + 0.2 M NaCl	100	100%
(1) 0.5 M NaCl	100	0.1
(2) 1.0 M NaCl	99.7	<0.01
(3) 8 M Urea	100	<0.01
(4) 6 M guanidine-HCl	95.2	<0.01
(5) 99% formamide	94.7	<0.01
(6) 2 mM Biotin	97.6	<0.01

TABLE I-continued

SELECTIVE RETENTION OF BIOTINIZED DNA ON AVIDIN-SEPHAROSE

Eluent	% DNA Retained on Resin	
	Bio-DNA (1%)	T-DNA
(7) 50 mM NaOH	89.5	<0.01

TABLE II

Affinity Chromatography of Iminobiotin-dUTP and Iminobiotinized - DNA on Streptavidin-Sepharose

Eluent	% Retained on SA-Sepharose		
	T-DNA	^{3}H—IB-dUTP	IB-DNA
Load - 10 mM Tris-HCl, 8.3 50 mM NaCl	8.7	100	99.7
(1) 0.1 M NaCl	<0.1	100	99.7
(2) 1.0 M NaCl	<0.01	100	99.4
(3) 8 M Urea	<0.01	97.5	98.5
(4) 6 M guanidine-HCl	<0.01	97.0	97.0
(5) 50 mM NH_4—acetate, pH 4.0	<0.01	<0.01	96.5
(6) 50 mM NH_4—acetate, pH 4.0 2 mM biotin	<0.01	<0.01	<0.01

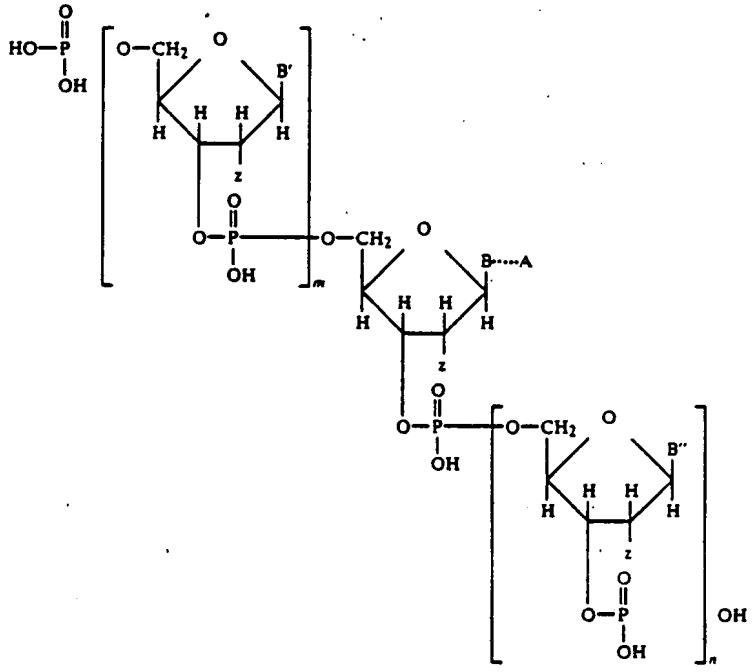
TABLE III

SELECTIVE IMMUNOPRECIPITATION OF BIO-DNA WITH ANTI-BIOTIN IgG and *STAPH AUREUS*

DNA*	Antibody	CPM in Immuno ppt.	CPM in Supernatant
T-DNA	—	70	4867
T-DNA	Anti-Bio IgG	87	5197
T-DNA	Non-immune IgG	55	5107
Bio-DNA	—	53	3886
Bio-DNA	Anti-Bio IgG	3347	736
Bio-DNA	Non-immune IgG	60	3900

*N.T. pBR-322 DNA, ^{32}p-labeled; 1% Biotin substitution. Specific activity, 2 × 10^7 cpm/μg Biotin detection 0.001–0.01 pmoles.

Thus, it is possible to prepare novel compounds having the structure:



14, it is preferred to operate in the range from about 4 to 8. This is especially true when dealing with unstable compounds such as nucleoside polyphosphates, polynucleotides, and nucleotide coenzymes which are hydrolyzed at pH's outside this range. Similarly, it is preferred to operate at a temperature in the range from about 20° C. to 30° C. to avoid possible decomposition of labile organic substrates. However, the reactions can be carried out at temperatures from about 5° C. to 100° C. As is usual with chemical reactions, higher temperatures promote the reaction rate and lower temperatures retard it. Thus, in the temperature range from 5° C. to 100° C., the optimum reaction time may vary from about 10 minutes to 98 hours. In the preferred temperature range, reaction times normally vary from about 3 to 24 hours.

The preferred procedure for maintaining the pH in the desired range is through the use of buffers. A variety of buffers can be employed. These include, for example, sodium or potassium acetate, sodium or potassium citrate, potassium citrate-phosphate, tris-acetate and borate-sodium hydroxide buffers. The concentration of buffer, when employed, can vary over a wide range, up to about 2.0 molar.

While a particular advantage of the mercuration and palladium catalyzed addition reactions is that they can be carried out in water, small amounts of an organic solvent can be usefully included as a solubility aid. The organic solvents usually chosen are those which are miscible with water. These may be selected from ethers, alcohols, esters, ketones, amides, and the like such as methanol, ethanol, propanol, glycerin, dioxane, acetone, pyridine and dimethylformamide. However, since it has been observed that the presence of alcohols, such as methanol, often results in alkoxy-addition across the olefin double bond, any organic solvent used as a solubility aid should be chosen carefully. Introduction of alkoxy substituents to the α- or β-exocyclic carbon atoms often results in the production of compounds which are utilized much less efficiently as enzyme substrates.

Although various mercuric salts may be utilized, the presently preferred salt is mercuric acetate. Also, as indicated previously, the compounds may be prepared by first adding a linker arm and then the moiety A, or by adding a linker arm to which A is already attached. Thus, the chemical moiety represented by the formula . . . N may be any one of the numerous entities which ultimately result in production of the desired compounds.

Examples include $-CH{=}CH-CH_2-NH_2$,

$$-CH{=}CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH_2,$$

$-CH{=}CH-CH_2-NH-$biotin, and

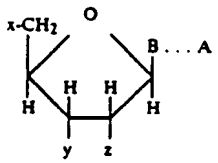
$$-CH{=}CH_2-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH-\text{iminobiotin.}$$

The amounts of the reactants employed in these reactions may vary widely. However, in general the amounts of unmercurated compound, mercurated compound, and palladium-containing compound will be substantially stoichiometric whereas the mercuric salt and compound . . . N will be present in molar excess, e.g. 5-20 moles of . . . N or of mercuric salt per mole of mercurated compound or unmercurated compound, respectively. In practice, amounts will vary depending upon variations in reaction conditions and the precise identity of the reactants.

Having the biotin probe directly attached to nucleotide derivatives that are capable of functioning as enzyme substrates offers considerable versatility, both in the experimental protocols that can be performed and in the detection methods (microscopic and non-microscopic) that can be utilized for analysis. For example, biotin nucleotides can be introduced into polynucleotides which are in the process of being synthesized by cells or crude cell extracts, thus making it possible to detect and/or isolate nascent (growing) polynucleotide chains. Such a procedure is impossible to do by any direct chemical modification method. Furthermore, enzymes can be used as reagents for introducing probes such as biotin into highly selective or site-specific locations in polynucleotides; the chemical synthesis of similar probe-modified products would be extremely difficult to achieve at best.

The synthesis of nucleotides containing biotin or iminobiotin was achieved as detailed in the examples set forth hereinafter. Pyrimidine nucleoside triphosphates containing either of these probes attached to the C-5 carbon atom were good to excellent substrates for a wide variety of purified nucleic acid polymerases of both prokaryotic and eukaryotic origin. These include DNA polymerase I of *E. coli*, bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine (A-9) and human (HeLa) cells, and the DNA polymerase of *Herpes simplex* virus. Confirming data were obtained with *E. coli* DNA polymerase I using either the nick-translation condition of Rigby, et al. (P. W. J. Rigby, M. Dieckmann, C. Rhodes and P. Berg, J. Mol. Biol. 113, 237, 1977) or the gap-filling reaction described by Bourguignon et al. (G. J. Bourguignon, P. J. Tattersall and D. C. Ward, J. Virol. 20, 290, 1976). Bio-dUTP has also been found to function as a polymerase substrate both in CHO cells, permeabilized by treatment with lysolecithin according to the method of Miller, et al. (M. R. Miller, J. C. Castellot, Jr. and A. B. Pardee, Exp. Cell Res. 120, 421, 1979) and in a nuclear replication system prepared from *Herpes simplex* infected BHK cells. Although biotinyl ribonucleoside triphosphates were found to function as substrates for the RNA polymerases of *E. coli* and bacteriophage T7, they are not utilized as efficiently as their deoxyribonucleotide triphosphate counterparts. Indeed, they are incorporated poorly, if at all, by the eukaryotic RNA polymerases examined (HeLa cell RNA polymerase III, calf thymus RNA polymerase II and mouse cell RNA polymerase II). While this limited range of substrate function does restrict the utility in some in vivo or in vitro transcription studies, biotin-labeled RNA probes can be prepared enzymatically from DNA templates using *E. coli* or T7 RNA polymerases or by 3' end-labeling methods using RNA ligase with compounds such as biotinyl-pCp. The AA- and NAGE-derivatives of UTP are, however, substrates for the eukaryotic RNA polymerases mentioned above. With the availability of antibodies to these analogs, the isolation of nascent transcripts by immunological or affinity procedures should be feasible.

The enzymatic polymerization of nucleotides containing biotin or iminobiotin substituents was not monitored directly, since neither of these probes were radiolabeled. However, two lines of experimental evidence

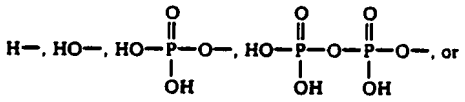


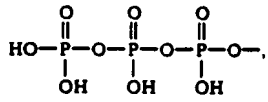
wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

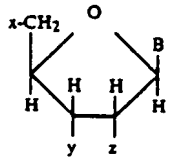
wherein each of x, y, and z represents



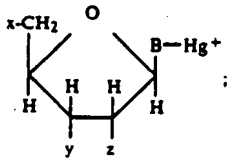


can be prepared by:

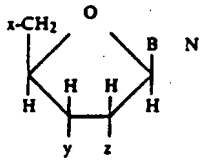
(a) reacting a compound having the structure:



with a mercuric salt in a suitable solvent under suitable conditions so as to form a mercurated compound having the structure:



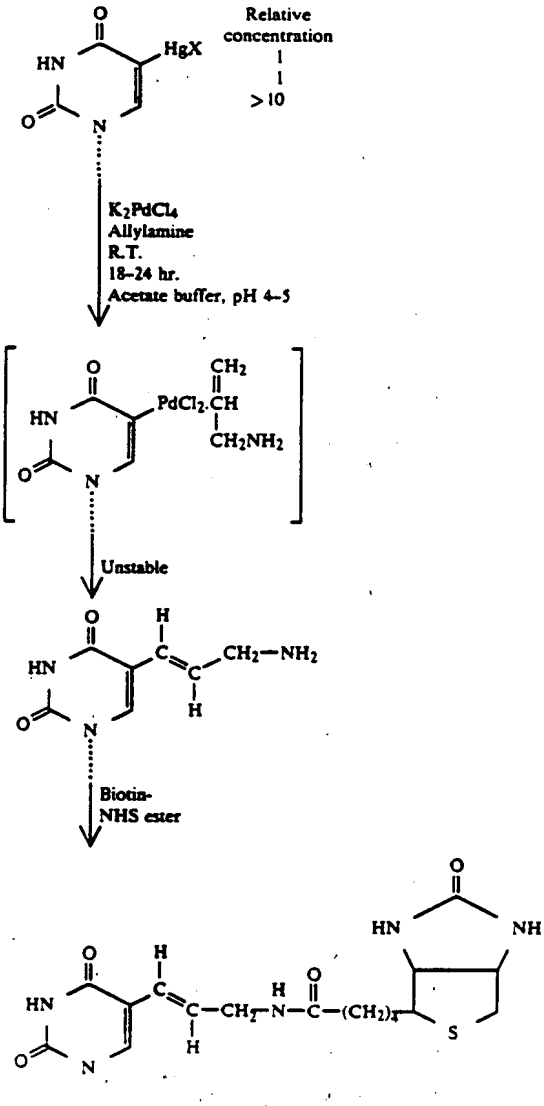
(b) reacting said mercurated compound with a chemical moiety reactive with the $-Hg^+$ portion of said mercurated compound and represented by the formula . . . N, said reaction being carried out in an aqueous solvent and in the presence of K_2PdCl_4 under suitable conditions so as to form a compound having the structure:



wherein N is a reactive terminal functional group or is A; and

(c) recovering said compound as said modified nucleotide when N is A, or when N is a reactive terminal group, reacting said compound with a compound having the structure M—A, wherein M represents a functional group reactive with N in an aqueous solvent under suitable conditions, so as to form said modified nucleotide, which is then recovered.

The following schema is illustrative:



Although the reactions can be carried out at hydrogen ion concentrations as low as pH 1, or as high as pH

relative to B. The presence of such an α-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double-helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide.

It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure $-CH_2-NH-$, since such linkages are easily formed utilizing any of the well known amine modification reactions. Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxyl-1-propyl)ether groups have the formulae $-CH=CH-CH_2-NH-$ and

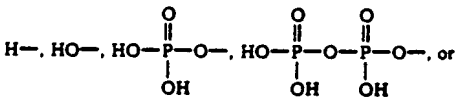
$$-CH=CH-CH_2-O-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-NH-,$$

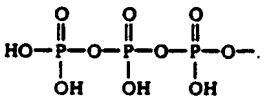
respectively.

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities such as thiol, carboxylic acid, and epoxide functionalities.

The linkage groups are attached at specific positions, namely, the 5-position of a pyrimidine, the 8-position of a purine, or the 7-position of a deazapurine. As indicated previously, substitution at the 8-position of a purine does not produce a modified nucleotide which is useful in all the methods discussed herein. It may be that the 7-position of a purine, which is occupied by a nitrogen atom, could be the point of linkage attachment. However, the chemical substitution methods employed to date and discussed herein are not suitable for this purpose.

The letters x, y, and z represent groups attached to the 5', 3', and 2' positions of the sugar moiety. They may be any of



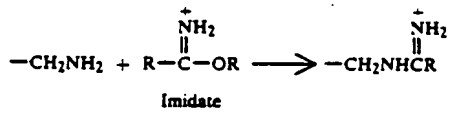


Although conceivable, it is unlikely that all of x, y, and z will simultaneously be the same. More likely at least one of x, y, and z will be a phosphate-containing group, either mono-, di-, or tri-phosphate and at least one will be HO— or H—. As will be readily appreciated, the most likely identity of z will be HO— or H— indicating ribonucleotide or deoxyribonucleotide, respectively. Examples of such nucleotides include 5'-ribonucleoside monophosphates, 5'-ribonucleoside diphosphates, 5'-ribonucleoside triphosphates, 5'-deoxyribonucleoside monophosphates, 5'-deoxyribonucleoside diphosphates, 5'-deoxyribonucleoside triphosphates, 5'p-ribonucleoside-3'p, and 5'p-deoxyribonucleoside-3'p. More specific examples include modified nucleotides of this type in which A is biotin or iminobiotin, the chemical linkage is $-CH=CH-CH_2-NH-$ or

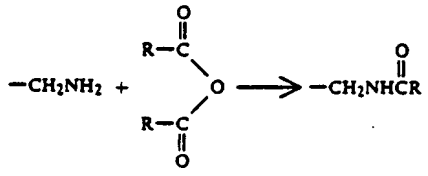
$$-CH=CH-CH_2-O-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-NH-,$$

and B is uracil or cytosine.

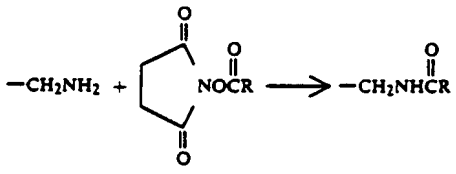
The general synthetic approach adopted for introducing the linker arm and probe moiety onto the base is discussed hereinabove. (See especially, J. L. Ruth and D. E. Bergstrom, J. Org. Chem., 43, 2870, 1978; D. E. Bergstrom and M. K. Ogawa, J. Amer. Chem. Soc. 100, 8106, 1978; and C. F. Bigge, P. Kalaritis, J. R. Deck and M. P. Mertes, J. Amer. Chem. Soc. 102, 2033, 1980.) However, the olefin substituents employed herein have not been used previously. To facilitate attachment of probe moiety A, it has been found particularly desirable to employ olefins with primary amine functional groups, such as allylamine [AA] or allyl-(3-amino-2-hydroxy-1-propyl)ether [NAGE], which permit probe attachment by standard amine modification reactions, such as,



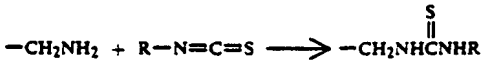
Imidate



Anhydride



NHS—ester
(N—hydroxysuccinimide)



Isothiocyanate

Epoxide

Because of ease of preparation it has been found preferable to use NHS-esters for probe addition. However, olefin linker arms with other modifiable functional groups, such as thiols, carboxylic acids, epoxides, and the like, can also be employed. Furthermore, both linker arm and probe can be added in a single-step if deemed desirable.

Specifically, modified nucleotides having the structure:

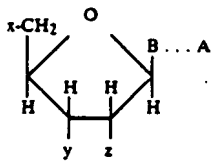
"linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.

Fifth, the physical and biochemical properties of polynucleotides containing small numbers of probe substituents should not be significantly altered so that current procedures using radioactive hybridization probes need not be extensively modified. This criterion must be satisfied whether the probe is introduced by enzymatic or direct chemical means.

Finally, the linkage that attaches the probe moiety should withstand all experimental conditions to which normal nucleotides and polynucleotides are routinely subjected, e.g., extended hybridization times at elevated temperatures, phenol and organic solvent extraction, electrophoresis, etc.

All of these criteria are satisfied by the modified nucleotides described herein.

These modified nucleotides have the structure:

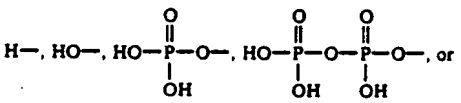


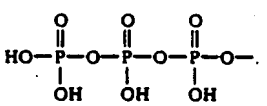
wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or 7-deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a linkage group joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y and z represents





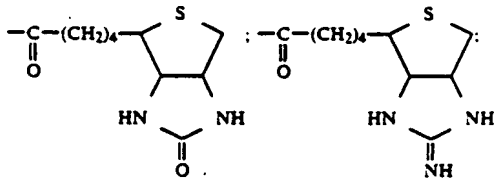
These compounds are widely useful as probes in biomedical research and recombinant DNA technology.

Although in principal all compounds encompassed within this structural formula may be prepared and used in accordance with the practices of this invention, certain of the compounds are more readily prepared or used or both, and therefore are presently preferred.

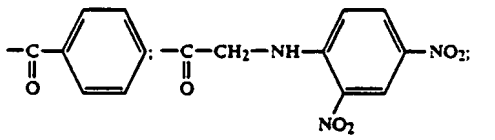
Thus, although purines, pyrimidines and 7-deazapurines are in principal useful, pyrimidines and 7-deazapurines are preferred since purine substitution at the 8-position tends to render the nucleotides ineffective as polymerase substrates. Thus, although modified purines are useful in certain respects, they are not as generally useful as pyrimidines and 7-deazapurines. Moreover, pyrimidines and 7-deazapurines useful in this invention must not be naturally substituted at the 5- or 7-positions, respectively. As a result, certain bases such as thymine, 5-methylcytosine, and 5-hydroxymethylcytosine are not useful. Presently preferred bases are cytosine, uracil, deazaadenine and deazaguanine.

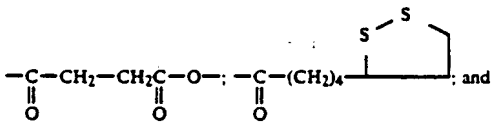
A may be any moiety which has at least three carbon atoms and is capable of forming a detectable complex with a polypeptide when the modified nucleotide is incorporated into a double-stranded duplex containing either deoxyribonucleic or ribonucleic acid.

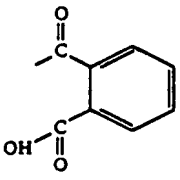
A therefore may be any ligand which possesses these properties, including haptens which are only immunogenic when attached to a suitable carrier, but are capable of interracting with appropriate antibodies to produce complexes. Examples of moieties which are useful include:



biotin iminobiotin



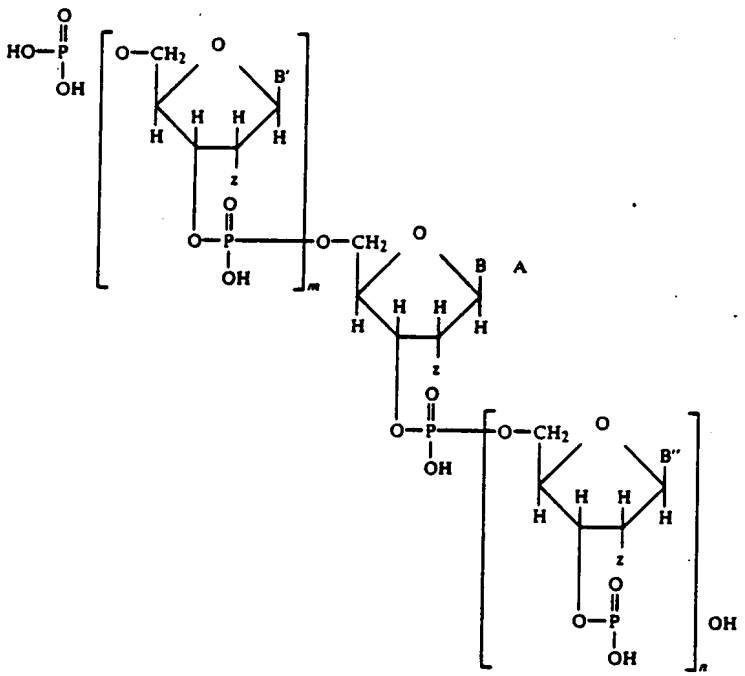




Of these the preferred A moieties are biotin and iminobiotin.

Moreover, since aromatic moieties tend to intercalate into a base-paired helical structure, it is preferred that the moiety A be nonaromatic. Also, since smaller moieties may not permit sufficient molecular interaction with polypeptides, it is preferred that A be at least C_5 so that sufficient interaction can occur to permit formation of stable complexes. Biotin and iminobiotin satisfy both of these criteria.

The linkage or group joining moiety A to base B may include any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds. However, it is generally preferred that the chemical linkage include an olefinic bond at the α-position



wherein each of B, B', and B" represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C1'-position of the sugar moiety, provided that whenever B, B', or B" is purine or 7-deazapurine, it is attached at the N^9-position of the purine or 7-deazapurine, and whenever B, B', or B" is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded duplex formed with a complementary ribonucleic or deoxyribonucleic acid molecule.

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine;

wherein z represents H- or HO-; and

wherein m and n represent integers from 0 up to about 100,000.

These compounds can be prepared by enzymatic polymerization of a mixture of nucleotides which include the modified nucleotides of this invention. Alternatively, nucleotides present in oligo- or polynucleotides may be modified using chemical methods.

Nucleotides modified in accordance with the practices of this invention and oligo- and polynucleotides into which the modified nucleotides have been incorporated may be used as probes in biomedical research, clinical diagnosis, and recombinant DNA technology. These various utilities are based upon the ability of the molecules to form stable complexes with polypeptides which in turn can be detected, either by means of properties inherent in the polypeptide or by means of detectable moieties which are attached to, or which interact with, the polypeptide.

Some uses include detecting and identifying nucleic acid-containing etiological agents, e.g. bacteria and viruses; screening bacteria for antibiotic resistance; diagnosing genetic disorders, e.g. thalassemia and sickle cell anemia; chromosomal karyotyping; and identifying tumor cells.

DETAILED DESCRIPTION OF THE INVENTION

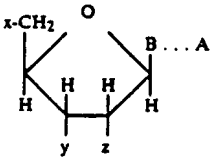
Several essential criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound must contain a substituent or probe that is unique, i.e., not normally found associated with nucleotides or polynucleotides. Second, the probe must react specifically with chemical or biological reagents to provide a sensitive detection system. Third, the analogs must be relatively efficient substrates for commonly studied nucleic acid enzymes, since numerous practical applications require that the analog be enzymatically metabolized, e.g., the analogs must function as substrates for nucleic acid polymerases. For this purpose, probe moieties should not be placed on ring positions that sterically, or otherwise, interfere with the normal Watson-Crick hydrogen bonding potential of the bases. Otherwise, the substituents will yield compounds that are inactive as polymerase substrates. Substitution at ring positions that alter the normal "anti" nucleoside conformation also must be avoided since such conformational changes usually render nucleotide derivatives unacceptable as polymerase substrates. Normally, such considerations limit substitution positions to the 5-position of a pyrimidine and the 7-position of a purine or a 7-deazapurine.

Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic acid hybridization methodologies. To satisfy this criterion, it is preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or

These new nucleotide derivatives can be prepared relatively inexpensively by chemical procedures which have been developed and standarized as discussed more fully hereinafter. More significantly, since neither the nucleotide probes of this invention nor the protein reagents employed with them are radioactive, the compounds can be prepared, utilized, and disposed of, without the elaborate safety procedures required for radioisotopic protocols. Moreover, these nucleotide derivatives are chemically stable and can be expected to have functional shelf-lives of several years or more. Finally, these compounds permit the development of safer, more economical, more rapid, and more reproducible research and diagnostic procedures.

SUMMARY OF THE INVENTION

Compounds having the structure:

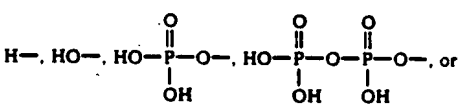


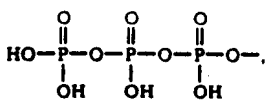
wherein B represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the C$^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y, and z represents



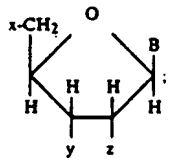


are widely used as probes in biomedical research and recombinant DNA technology.

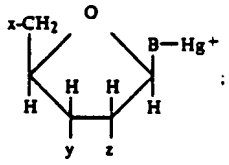
Particularly useful are compounds encompassed within this structure which additionally have one or more of the following characteristics: A is non-aromatic; A is at least C_5; the chemical linkage joining B and A includes an α-olefinic bond; A is biotin or iminobiotin; and B is a pyrimidine or 7-deazapurine.

These compounds may be prepared by a process which involves:

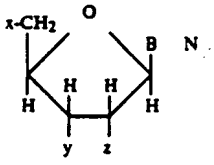
(a) reacting a compound having the structure:



with a mercuric salt in a suitable solvent under suitable conditions so as to form a mercurated compound having the structure:



(b) reacting said mercurated compound with a chemical moiety reactive with the —Hg$^+$ portion of said mercurated compound and represented by the formula . . . N, said reaction being carried out in an aqueous solvent and in the presence of K_2PdCl_4 under suitable conditions so as to form a compound having the structure:



wherein N is a reactive terminal functional group or is A; and

(c) recovering said compound as said modified nucleotide when N is A, or when N is a reactive terminal group, reacting said compound with a compound having the structure M-A, wherein M represents a functional group reactive with N in an aqueous solvent under suitable conditions so as to form said modified nucleotide, which is then recovered.

This invention also provides compounds having the structure:

MODIFIED NUCLEOTIDES AND METHODS OF PREPARING AND USING SAME

This application is a continuation of copending U.S. patent application Ser. No. 255,223 filed Apr. 17, 1981 now abandoned.

BACKGROUND OF THE INVENTION

Many procedures employed in biomedical research and recombinant DN technology rely heavily on the use of nucleotide or polynucleotide derivatives radioactively labeled with isotopes of hydrogen (^{3}H), phosphorus (^{32}P), carbon (^{14}C), or iodine (^{125}I). Such radioactive compounds provide useful indicator probes that permit the use to detect, monitor, localize, or isolate nucleic acids and other molecules of scientific or clinical interest, even when present in only extremely small amounts. To date, radioactive materials have provided the most sensitive, and in many cases the only, means to perform many important experimental or analytical tests. There are, however, serious limitations and drawbacks associated with the use of radioactive compounds. First, since personnel who handle radioactive material can be exposed to potentially hazardous levels of radiation, elaborate safety precautions must be maintained during the preparation, utilization, and disposal of the radioisotopes. Secondly, radioactive nucleotides are extremely expensive to purchase and use, in large part due to the cost of equipment and manpower necessary to provide the appropriate safeguards, producer-/user health monitoring services, and waste-disposal programs. Thirdly, radioactive materials are often very unstable and have a limited shelf-life, which further increases usage costs. This instability results from radiolytic decomposition, due to the destructive effects associated with the decay of the radioisotope itself, and from the fact that many isotopes (e.g. ^{32}P and ^{125}I) have half-lives of only a few days.

It is known that haptens can combine with antibodies, but can initiate an immune response only if bound to a carrier. This property can be exploited in detection and identification testing.

It is also known that biotin and iminobiotin strongly interact with avidin, a 68,000 dalton glycoprotein from egg white. This interaction exhibits one of the tightest, non-covalent binding constants ($K_{dis}=10^{-15}$) seen in nature. If avidin is coupled to potentially demonstrable indicator molecules, including fluorescent dyes, e.g. fluorescein or rhodamine; electron-dense reagents, e.g. ferritin, hemocyanin, or colloidal gold; or enzymes capable of depositing insoluble reaction products, e.g. peroxidase or alkaline phosphatase, the presence, location, or quantity of a biotin probe can be established. Although iminobiotin binds avidin less tightly than biotin, similar reactions can be used for its detection. Moreover, the reversibility of the iminobiotin-avidin interaction, by decreasing solution pH, offers significant advantages in certain applications.

The specificity and tenacity of the biotin-avidin complex has been used in recent years to develop methods for visually localizing specific proteins, lipids, or carbohydrates on or within cells (reviewed by E. A. Bayer and M. Wilchek in Methods of Biochemical Analysis, 26, 1, 1980). Chromosomal location of RNA has been determined by electron microscopy using a biotinized protein, cytochrome C, chemically cross-linked to RNA as a hybridization probe. The site of hybridization was visualized through the binding of avidin-ferritin or avidin-methacrylate spheres mediated by the avidin-biotin interaction. (J. E. Manning, N. D. Hershey, T. R. Broker, M. Pellegrini, H. K. Mitchell, and N. Davidson, Chromosoma, 53, 107, 1975; J. E. Manning, M. Pellegrini, and N. Davidson, Biochemistry, 61, 1364, 1977; T. R. Broker, L. M. Angerer, P. H. Yen, N. D. Hersey, and N. Davidson, Nucleic Acid Res., 5, 363, 1978; A Sodja and N. Davidson, Nucleic Acid Res., 5, 383, 1978.) This approach to the detection of polynucleotide sequences, although successful in the specialized cases examined which were highly reitterated sequences, is not of general utility for analysis of polynucleotides present in single or low copy number.

Moreover, methods for attaching chemical moieties to pyrimidine and purine rings are known. Several years ago a simple and rapid acetoxymercuration reaction was developed for introducing covalently bound mercury atoms into the 5-position of the pyrimidine ring, the C-8 position of the purine ring or the C-7 position of a 7-deazapurine ring, both in nucleotides and polynucleotides. (R. M. K. Dale, D. C. Livingston and D. C. Ward, Proc. Natl. Acad. Sci. U.S.A., 70, 2238, 1973; R. M. K. Dale, E. Martin, D. C. Livingston and D. C. Ward, Biochemistry, 14, 2447, 1975.) It was also shown several years ago that organomercurial compounds would react with olefinic compounds in the presence of palladium catalysts to form carbon-carbon bonds (R. F. Heck, J. Am. Chem. Soc., 90, 5518, 1968; R. F. Heck, Ibid., 90, 5526, 1968; R. F. Heck, Ibid., 90, 5531, 1968; R. F. Heck, Ibid., 90, 5535, 1968; and R. F. Heck, J. Am. Chem. Soc. 91, 6707, 1969.) Bergstrom and associates (J. L. Ruth and D. E. Berstrom, J. Org. Chem., 43, 2870, 1978; and D. E. Bergstrom and M. K. Ogawa, J. Am. Chem. Soc., 100, 8106, 1978) and Bigge, et al. (C. F. Bigge, P. Kalaritis, J. R. Deck and M. P. Mertes, J. Am. Chem. Soc., 102, 2033, 1980) have recently applied this reaction scheme in the synthesis of C-5 substituted pyrimidine nucleotide compounds.

Finally, it is known that antibodies specific for modified nucleotides can be prepared and used for isolating and characterizing specific constituents of the modified nucleotides. (T. W. Munns and M. K. Liszewski, Progress in Nucleic Acid Research and Molecular Biology, 24, 109, 1980.) However, none of the antibodies prepared to date against naturally occurring nucleotides have been shown to react with their nucleotide determinant when it exists in a double-stranded RNA or DNA duplex or when in DNA-RNA hybrid molecules.

To circumvent the limitations of radioactively labeled probes or previously utilized chemical and biological probes, a series of novel nucleotide derivatives that contain biotin, iminobiotin, lipoic acid, and other determinants attached covalently to the pyrimidine or purine ring have been synthesized. These nucleotide derivatives, as well as polynucleotides and coenzymes that contain them, will interact specifically and uniquely with proteins such as avidin or antibodies. The interaction between modified nucleotides and specific proteins can be utilized as an alternative to radioisotopes for the detection and localization of nucleic acid components in many of the procedures currently used in biomedical and recombinant-DNA technologies. Methods employing these modified nucleotide-protein interactions have detection capacities equal to or greater than procedures which utilize radioisotopes and they often can be performed more rapidly and with greater resolving power.

OTHER PUBLICATIONS

T. W. Munns and M. K. Liszewski, "Antibodies Specific for Modified Nucleotides: An Immunochemical Approach for the Isolation and Characterization of Nucleic Acids," in *Progress in Nucleic Acid Research and Molecular Biology*, ed. W. E. Cohn, Academic Press, 24, pp. 109–165 (1980).

T. Nelson and D. Brutlag, "Addition of Homopolymers to the 3'–ends of Duplex DNA with Terminal Transferase," *Methods in Enzymology* (vol. 68) *Recombinant DNA*, ed. R. Wu, pp. 41–50.

M. Pellegrini et al., "Application of the Avidin–Biotin Method of Gene Enrichment to the Isolation of Long Double-Stranded DNA Containing Specific Gene Sequences", *Nucl. Acids Res.*, 4, pp. 2961–2973 (1977).

J. Reiser et al., "Transfer of Small DNA Fragments from Polyacrylamide Gels to Diazobenzyloxymethyl Paper and Detection by Hybridization with DNA Probes," *Biochemical and Biophysical Research Communications*, 85, pp. 1104–1112 (1978).

P. W. J. Rigby et al., "Labeling Deoxyribonucleic Acid to High Specific Activity In Vitro by Nick Translation with DNA Polymerase I," *J. Mol. Biol.*, 113, pp. 237–251 (1977).

G. T. Rudkin and B. D. Stollar, "High Resolution Detection of DNA–RNA Hybrids *in situ* by Indirect Immunofluorescence," *Nature*, 265, pp. 472–473 (1977).

J. L. Ruth and D. E. Bergstrom, "C-5 Substituted Pyrimidine Nucleosides, 1. Synthesis of C-5 Allyl, Propyl, and Propenyl Uracil and Cytosine Nucleosides via Organopalladium Intermediates," *J. Org. Chem.*, 43, pp. 2870–2876 (1978).

M. So, "Characterization of an *Escherichia coli* Plasmid Encoding for the Synthesis of Heat–Labile Toxin: Molecular Cloning of the Toxin Determinant", *Infection and Immunity*, 21, pp. 405–41C (1978).

A. Sodja and N. Davidson, "Gene Mapping and Gene Enrichment by the Avidin–Biotin Interaction: Use of Cytochrome–C as a Polyamine Bridge," *Nucl. Acids Res.*, 5, pp. 383–399 (1978).

Manning et al., A New Method of In Situ Hybridization, Chromosoma 53, 107–117 (1975).

Stryer, Biochemistry, pp. 348, 512, 589 and 596 (2nd Ed., 1981).

Langer et al., Enzymatic Synthesis of Biotin-Labeled Polynucleotides, Proc. Natl. Acad. Sci. USA 78(11), 6633–37 (1981).

Guesdon et al., The Use of Avidin–Biotin Interaction in Immunoenzymatic Techniques, J. Histochem. Cytochem. 27(8), 1131–39 (1979).

PATENT SPECIFICATION

(11) 1 564 578

(21) Application No. 47957/77 (22) Filed 17 Nov. 1977 (19)
(31) Convention Application No. 51354 (32) Filed 28 Jan. 1977 in
(33) Israel (IL)
(44) Complete Specification Published 10 Apr. 1980
(51) INT. CL.³ G01N 31/06 // 33/56
(52) Index at Acceptance
G1B BA BR

(54) COLUMN CHROMATOGRAPHY SPECIFIC BINDING ASSAY METHOD AND TEST KIT

(71) We, AMES-YISSUM LTD., a Corporation organised and existing under the laws of the State of Israel, of Science Based Industries Campus, Har Hotzvim, Sanhedria Murhevet, Jerusalem, Israel, do hereby declare the invention for which we pray that a Patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

BACKGROUND OF THE INVENTION

1. *FIELD OF THE INVENTION*

This invention relates to the quantitative determination of substances in or characteristics of liquid media, including body fluids such as serum, based on specific binding assay techniques. In particular, the invention is directed to the detection of antigens or haptens based on immunoassay techniques involving the use of labeled reagents, such as radiolabeled reagents. The present invention provides an improved method of performing the separation of bound- and free-label inherent in heterogeneous specific binding assays.

2. *DESCRIPTION OF THE PRIOR ART*

A living system is able to detect, recognize and respond to the presence of foreign material (antigen) such as protein, virus, bacteria, and so forth, within that system. This response takes, *inter alia*, the form of producing an antibody specific for the particular antigen. There then occurs a specific reaction between the antibody and the antigen to form a complex. An antibody once produced is also capable of binding a hapten, i.e., a relatively small and simple compound which may be the determinant group of a given antigen, which hapten is capable of binding with the specific antibody but incapable itself of giving rise to the production of an antibody, unless it is bound to an antigenic carrier.

The binding interaction between an antigen or a hapten and its antibody is specific and sensitive. Other types of materials that participate in similar specific and sensitive binding interactions are enzymes and their substrates; materials such as hormones, vitamins, metabolites and pharmacological agents, and their receptors and binding substances; and other substances known in the science. These specific and sensitive binding reactions have given rise to a rapidly emerging analytical technique known as the specific binding assay technique. In one such type of assay method, the substance, or group of substances, to be determined (herein referred to as "ligand") in a liquid sample is placed in competition with a labeled form of the ligand or of a binding analog thereof for binding to a binding reagent. Where a radioactive label is used and the binding reagent is an antibody, the method is known as a radioimmunoassay method. Recently, several alternative labeling materials have been reported for replacement of radioisotopes, including enzymes, coenzymes, enzyme substrates, enzyme modulators such as inhibitors and allosteric effectors, fluorescent molecules, luminescent molecules, and others. For illustrative purposes, the discussion which follows describes one particular type of specific binding assay technique, a competitive binding radioimmunoassay technique.

This system consists of antigen or hapten labeled with a radioactive marker, unlabeled native antigen (in the test sample) and specific antibody whereby there is competition between the unlabeled antigen and the labeled antigen for binding to a limited amount of antibody. Hence, the greater the concentration of unlabeled antigen from the test sample in the system, the less the labeled antigen will be bound by the antibody. This may be diagrammatically represented as follows:

LABELED ANTIGEN + SPECIFIC ANTIBODY + UNLABELED ANTIGEN

$$^{*}Ag \quad Ab \quad Ag$$

$$\downarrow$$

LABELED AND UNLABELED

ANTIGEN–ANTIBODY COMPLEX

$$(^{*}Ag\text{-}Ab) + (Ag\text{-}Ab)$$

If the concentration of labeled antigen and antibody is fixed and the only variable is the level of unlabeled antigen, it becomes possible to establish an assay system for measuring the unknown level of unlabeled antigen by physically separating the antigen-antibody complex from the remaining free antigen (both labeled and unlabeled). The radioactivity of the unknowns is compared with a standard curve plotting of the values given by a range of known amounts of the antigen treated in the same manner.

There are many known procedures for separating the free unbound antigen or hapten from the complex antigen-antibody. One method known as chromatoelectrophoresis combines techniques of paper chromatography and paper electrophoresis. Paper with a high affinity for the free antigen (such as Whatman 3 MM, Whatman 3 MC and DEAE paper) are used as carriers. ("Whatman" is a registered Trade Mark.) While this technique is discriminative and has been used in the assay of insulin, growth hormone, glucagon, parathyroid hormone, thyroid stimulating hormone and other peptide hormones, it has a number of prominent disadvantages which limits its use. A limited amount of material may be applied to the adsorbent, and the separation is both laborious and time-consuming.

Another known procedure, which has been proposed for the assay of the above-mentioned peptide hormones, makes use of ascending paper-wick chromatography, e.g., on Whatman 3MC and DE-cellulose paper or on paper loaded with weak ion-exchange resins. Orskov, *Scand. J. Clin. Lab. Invest. 20:* 297(1967). This method too has the disadvantage that the amount of sample which can be applied to the lower tip of the paperwick is comparatively small. It is further necessary in this technique to dry the paper (sometimes even twice) and to cut it before the counting, which is disadvantageous for applying the method to the assay of a large number of samples by mechanical means. It is also most significant that in more than a decade this known method has not been cited as being of any importance in the literature and has not been applied to the radioimmunoassay of relatively low-molecular weight haptens.

By another known method the antigen-antibody complex is precipitated by salts, organic material or solvents under conditions that do not affect the free antigen. Among the salts, materials and solvents used are: ethanol, acetone, sodium sulfate, ammonium sulfate, dioxane, trichloroacetic acid, and polyethylene glycol. The use of salts, solvents or organic materials has the advantage that the separation is immediate, and a second incubation is not necessary. However, the chemical precipitation technique may cause the co-precipitation of other proteins, often causing an incomplete separation of the two fractions.

There is also known the double antibody technique, which is widely used for the separation of the bound and the free antigen. By this method a second antibody that was raised against the first antibody is used to precipitate the primary antigen-antibody complex. For example, if the first antibody was raised in a rabbit then the second antibody may be an antiserum to rabbit gammaglobulin raised in goats. One disadvantage of this technique is that the use of a second antibody introduces an additional incubation. Specific binding assay methods employing a double antibody separation technique are described in U.S. Patents Nos. 3,839,153 and 3,872,225.

Furthermore, there are known various solid-phase techniques for the separation of free and bound antigen. These techniques make use of antibodies covalently bound or physically adsorbed to an insoluble matrix (immunosorbents), such as bentonite, cellulose, bromacetyl cellulose, the cross-linked dextrans (e.g. the product sold under the registered Trade Mark Sephadex), plastics (non-cross-linked polystyrene or polypropylene) beads, the products sold under the registered Trade Marks Sepharose and Enzacryl AA, and nitro-cellulose membranes. The formed antibody-antigen complex is held by the solid phase and the bound fraction is thus directly separated from the free fraction.

By yet another method the free (unbound) antigens are bound to adsorbents which then can be precipitated by centrifugation. Powdered talc (magnesium silicate), Kaolin (aluminum silicate), QUSO (registered Trade Mark for a brand of microgranules of silica), and cellulose powder, are some of the simple adsorbents used. Many separations are performed by using adsorbent charcoal coated with dextran. The dextran behaves rather like a sieve which allows the smaller molecules of free antigen to pass and these are then bound by the charcoal,

leaving the bound antigen in solution, after the charcoal has been removed by centrifugation or filtration.

It is also known to use ion exchange or other types of resins to bind free antigens by electrostatic forces and this method has been used so far mainly for the determination of small molecules such as thyroid hormones (T-3 and T-4). Examples of this type of methodology are described in U.S. Patents Nos. 3,659,104; 3,710,117 and 3,961,894.

One technique of this type used for the separation of the antigen-antibody complex from free antigen employs a column packed with material which preferentially adsorbs either the free antigen or the antigen-antibody complex. The incubated aqueous reaction mixture is applied to the head of such a column and the column is then eluted. The radioactivity of either the column or the eluate is then determined and the content of the antigen in the starting solution is calculated from the count.

In practice it has been found that this technique is somewhat cumbersome and not well suited for the rapid performance of a large number of radioimmunoassays with the aid of mechanical means. One of the reasons for this is that it is necessary to wash the non-adsorbed component completely out of the column which takes time and requires a relatively large amount of buffer solution.

It is an object of the present invention to provide an improved specific binding assay method in which the separation of the bound-species of the labeled component and the free-species thereof is accomplished in a novel manner which is more advantageous than the separation methods known in the art.

SUMMARY OF THE INVENTION

The present invention provides a specific binding assay method for determining a ligand in, or the ligand binding capacity of, a liquid medium, which comprises (A) when the said ligand is to be determined, combining the said liquid medium with an assay reagent means comprising (i) as labeled component, the said ligand or a binding analogue thereof, incorporated with a label and (ii) a binding agent for the said ligand; or when the ligand binding capacity of the said liquid medium suspected to contain a binding agent for the said ligand is to be determined, combining the said liquid medium with an assay reagent means comprising, as labeled component, the said ligand, or a binding analogue thereof, incorporated with a label, so as to form a binding reaction mixture having a bound-species as the said labeled component bound to the said binding agent and a free-species as the said labeled component not bound to the said binding agent; (B) separating the said bound-species and the said free-species by contacting at least a portion of the said binding reaction mixture a predetermined time after formation thereof with a column comprising an adsorbent material which is both selective for binding one of the said bound-species and free-species and capillarily absorbent relative to the said liquid medium so that the said portion of the binding reaction mixture is drawn into the said column by capillary action and the said bound-species and the said free-species are separated along the said column; and (C) measuring the said label in one of the separated species.

In preferred embodiment, the column of adsorbent is in the form of an elongated tube packed with a sufficient quantity of the adsorbent to effect complete take up of all of the binding reaction mixture upon contact of one end of such tube with such mixture. Preferably the adsorbent column is kept in a vertical position during the capillary absorption process resulting in an ascending chromatographic separation. To enhance separation of the bound- and free-species along the column, a volume of an inert liquid such as a buffer may be allowed to be absorbed into the adsorbent column after all of the reaction mixture has been absorbed.

The adsorbent used is selective for one of the bound- and free-species, usually the latter, and binds non-specifically therewith to substantially immobilize that species against the movement of the reaction mixture through the adsorbent column. The other species will of course be carried by the flow of the reaction mixture away from the beginning portion of the column where the immobilized species is, thereby effecting inherent separation of the bound- and free-species.

The great advantage of this separation technique is that the necessary separation step is reduced to the simple task of contacting the reaction mixture with the column adsorbent for a sufficient period of time to permit the necessary absorption of the liquid into the adsorbent. Further, the resulting column carrying the separated bound- and free-species is quite convenient for subsequent measuring steps, particularly where the label is radioactive. Even further, using the present column chromatography method, the mechanical steps of initiating the separation step and removing the separation device to a measuring location are readily adaptable to automation. An additional advantage is afforded when the label used is of a hazardous type, such as a radioactive label, since all of the label added in forming the reaction mixture ends up in a single, readily disposable device - the adsorbent column.

The present invention separation method is applicable generally to the specific binding

assay detection of ligands, such as the radioimmunoassay detection of antigens and haptens including thyroxine (T-4) and digoxin, and to the assay of sample binding capacity for various ligands, such as the serum binding capacity for triiodothyronine (T-3).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a cross-sectional view of a well of a counter in the course of performance of a measurement in accordance with a preferred embodiment of the invention;

Fig. 2 is an illustrative standard curve obtained by plotting the percent retention against known amounts of thyroxine using cross-linked polyvinyl alcohol columns;

Fig. 3 is an illustrative standard curve obtained by plotting the percent retention against known amounts of digoxin using cross-linked polyvinyl alcohol columns;

Fig. 4 is an illustrative standard curve obtained by plotting the percent retention against known amounts of thyroxine using cross-linked dextran columns;

Fig. 5 is an illustrative standard curve obtained by plotting the percent retention against known amounts of digoxin using cross-linked dextran columns;

Fig. 6 is an illustrative standard curve obtained by plotting the percent retention against known amounts of thyroxine using columns filled with formaldehyde treated starch;

Fig. 7 is an illustrative standard curve obtained by plotting the percent retention against known amounts of digoxin using starch filled columns; and

Fig. 8 is an illustrative standard curve obtained by plotting the percent retention against known amounts of digoxin using silica gel columns.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the context of this disclosure, the following terms shall be defined as follows: "ligand" is the substance, or group of substances, whose presence or the amount thereof in a liquid medium is to be determined; "binding agent for the ligand" is any substance, or group of substances, which has a specific binding affinity for the ligand to the exclusion of other substances; and "binding analog of the ligand" is any substance, or group of substances, which behaves essentially the same as the ligand with respect to the binding affinity of the binding agent for the ligand.

The separation of the bound- and free-species of the labeled component in specific binding assays by selective adsorption is well-known. According to the prior art methods, interaction between the binding reaction mixture and the adsorbent is effected either by addition to the reaction mixture of the adsorbent in a powder, bead, or strip form followed by physical removal of the adsorbent such as by centrifugation or filtration or by withdrawal of the strip adsorbent; or by percolating and washing the reaction mixture downwardly under influence of gravity through a bed of the adsorbent whereby the effluent emerging from the bed contains only one of the bound- and free-species of the labeled constituent.

The present invention affords an improved separation technique employing a selective adsorbent in that the reaction mixture is drawn into a column of the adsorbent material by capillary action. While under some circumstances it may be necessary or desirable to draw only a portion of the reaction mixture into the adsorbent column, normally the volume of the reaction mixture and the capacity of the column is selected so that the entire reaction mixture is drawn into the adsorbent column. Selective adsorption of the bound- or free-species occurs immediately upon contact between the reaction mixture and the adsorbent at the beginning portion of the adsorbent column. However, the non-adsorbed species moves on along the column with the advancing solvents in the reaction mixture. Upon completion of the movement of the reaction mixture into the column, substantially all of the selectively adsorbed species has become immobilized in the beginning end region of the column whereas the other species is found towards the other end of the column.

Enhancement of separation between the bound- and free-species can be accomplished by following the take up of the reaction mixture by exposure of the beginning end portion of the column to a volume of a liquid inert with respect to the selective adsorption of the one species at the beginning end portion but which acts as further solvent to carry the other species further into the adsorbent column. In the usual case, this liquid is water or an aqueous buffer solution.

Since numerous adsorbents selective for one of the bound- and free-species are well documented in the published literature, it does not serve any useful purpose to set forth an exhaustive list here. The adsorbent column is of a type which is capable of drawing the reaction mixture thereinto by capillary action. This is accomplished by using an adsorbent which is capillarily absorbent. Particularly useful adsorbents have been found to include cross-linked polyvinyl alcohol, cross-linked dextrans, starch, formaldehyde-treated starch and silica gel. These adsorbents are appropriately hydrophilic and inert to aqueous reaction mixtures, and can be selected to preferentially adsorb the free-species of commonly performed binding assays.

The adsorbent column is preferably in the form of a bed of adsorbent contained within an elongated tube and supported at least at one end by retainer means pervious to the reaction mixture. An example of a useful column device is a plastics tube (polypropylene or polystyrene) packed with a particulate form of the adsorbent held in place between porous disks of plastics, glass wool, or paper.

As a rule the volume of the reaction mixture used for the assay is small, e.g., of the order of 0.5 to 1 ml. The adsorbent column should be so dimensioned that the bulk of the non-adsorbed component becomes sufficiently removed from the bulk of the preferentially adsorbed component which remains at the beginning portion so as to enable the selective measurement of the label specified. For example, where the body of adsorbent material is enclosed in a tube, the inner diameter of the tube may range from 0.1 to 1.0 cm and will preferably be around 0.5 cm. The intake tip of such a tube will advantageously have a constriction adapted to hold a plug of porous material, e.g., of glass wool, which serves as a retainer for the absorbent while being pervious to the aqueous reaction mixture. A practical length for such a tube may be from 5 to 10 cm.

In principle, the label may be any of those known in the art as discussed above, however, it is most advantageous to use a label which can be measured in the adsorbent column without removal of the adsorbent. For this system, the adsorbent is held in containing means transparent to the labeling characteristic. The use of radioactive labels, particularly gamma-emitting isotopes, fits well into this scheme. The art is replete with teachings concerning the use of such radiolabels as ^{125}I, ^{131}I, ^{57}Co and so forth in binding assays. The present invention provides a separation means well suited for radioassays since the column device itself can be used in the measurement step and provides, upon the completion of the test, a disposable device containing all of the radioactivity used in the assay.

The selective measurement of the radioactivity of the intake region of the absorbent column is a simple operation due to the physical separation of the components on the column and the low radiation intensities of the radioactive labels preferably employed (mostly ^{125}I at intensities not exceeding 200 kcpm). As a rule, a counter for the measurement of radioactivity comprises a so-called well, i.e., a recess or socket adapted to receive a sample to be measured. Beyond the lateral wall of that well there is located at least one detector. Where the distance between the nonadsorbed component and the adsorbed one is larger than the depth of the well, the selective measurement occurs automatically upon the introduction of the column into the well with the intake region inwards. Where this is not the case and the depth of the well is larger than the distance between the two components, it is possible to shield off that portion of the column containing the nonadsorbed component with a radioactivity opaque absorbing material, e.g., lead, so that the detector responds only to the radioactivity of the intake portion of the column. Such a shield will be shaped and dimensioned in accordance with the specific geometry of the counter and the sample used. The shield can be in the form of a plate or, most preferably, a cylindrical tube having an inner diameter only slightly larger than the column device.

As shown in Fig. 1, well 1 of the counter is partially shielded by tubular metal shield 2, e.g., of lead, mounted in a removable manner. In Fig. 1, shield 2 is schematically shown in close proximity to the wall of well 1, but, as pointed out above, shield 1 should most preferably fit closely around test tube 4 containing the sample. Facing the unshielded portion of well 1 is detector 3. Into the well there is inserted test tube 4 in which the incubation was originally carried out and from which the reaction mixture was drawn into tube 5 packed with selective absorbent 6. Absorbent 6 is retained by two retainer plugs 7 and 8, e.g., of glass wool as shown. In consequence of the take up of the reaction mixture by absorbent 6 in tube 5, the liquid has ascended up to boundary 9 and in this way the bulk of the nonadsorbed component has been transported to the region of well 1 which is shielded by shield 2 while the adsorbed component is retained in the lower intake region of tube 5 in the non-shielded portion of the well. In this way only the radioactivity of the intake region is selectively measured. If it is desired to measure the total radioactivity of the material in tube 5, shield 2 is withdrawn.

Radioactivity counters are also known in which a detector is located below the bottom wall of the well, in addition to the lateral detectors. If such a counter is used in the method of the present invention, care must be taken to shield off said detector located below the bottom of the well, in order to prevent it from picking up counts from the aligned non-adsorbed component which has migrated up into the adsorbent column.

The present assay method may be applied to the detection of any ligand for which there is a specific binding partner. The ligand usually is a peptide, protein, carbohydrate, glycoprotein, steroid, or other organic molecule for which a specific binding partner exists in biological systems or can be synthesised. The ligand, in functional terms, is usually selected from antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites and pharmacological agents, and their receptors and binding substances. Specific examples of ligands which may be detected using the present invention are hormones such as

insulin, chorionic gonadotropin, thyroxine, triiodothyronine, and estriol; antigens and haptens such as ferritin, bradykinin, prostaglandins, and tumor specific antigens; vitamins such as biotin, vitamin B_{12}, folic acid, vitamin E, and ascorbic acid; metabolites such as 3', 5' adenosine monophosphate and 3', 5' guanosine monophosphate; pharmacological agents such as dilantin, digoxin, morphine, digitoxin, and barbiturates; antibodies such as microsomal antibody and antibodies to hepatitis and allergens; and specific binding receptors such as thyroxine binding globulin, avidin, intrinsic factor, and transcobalamin.

In a preferred embodiment, the detection of the ligand in the liquid medium, usually aqueous, is carried out by an improved radioimmunoassay method which comprises (A) mixing the said aqueous medium with a radiolabeled form of the said ligand or of a binding analogue thereof and with an antibody for said ligand and incubating the resulting reaction mixture for a predetermined period of time to form a bound-species of the said radiolabeled ligand or analogue wherein such is bound to the said antibody, (B) separating the said bound-species and the said free-species by contacting the said reaction mixture, after the said predetermined incubation period with a column comprising an adsorbent material which is selective for binding one of the said bound-species and free-species, capillarily absorbent relative to the said reaction mixture, and of sufficient volume to be capable of drawing all of the said reaction mixture thereinto by capillary action, so that the said bound-species and free-species are separated along the said column, and (C) measuring the radioactivity of one of the said separated species.

From the total radioactivity (total count) and the radioactivity of the intake portion of the column (partial count) measured after the separation of the components, the percent retention is calculated by the following formula:

$$\text{percent retention} = \frac{\text{partial count}}{\text{total count}} \times 100$$

For the determination of unknown quantities of the ligand, it is first necessary to perform a series of assays with varying known amounts of ligand, thereby to establish a standard percent retention versus concentration curve. This curve is then used to determine an unknown concentration from the percent retention calculated from radioactivity counts.

In principle, the total radioactivity is determined by the amount of radioactively labeled ligand or analog used for the preparation of the reaction mixture. However, to avoid inaccuracies due to imprecise application of the labeled component, it may be preferable to establish the total radioactivity experimentally. This may be done either before or after the selective determination of the radioactivity of the intake region of the adsorbent column. The total radioactivity may be determined after incubation and before the introduction of the absorbent column into the reaction mixture. Alternatively, where the selective counting of the intake tip region is effected by partial shielding of the counter well, it is also possible, in accordance with the present invention, to measure the total radioactivity after the separation of the components. To this end, after the counting of the intake region of the column is completed the shield is withdrawn from the well whereby the entire body becomes exposed to the counter which latter then responds to the total radioactivity.

The present invention also provides a method for determining the ligand binding capacity of a liquid medium. In such an assay, the liquid medium is suspected of containing a binding agent for the ligand. For example, the method according to the invention can be modified for the performance of the so called "T-3 Uptake Test". In this test, the thyroid hormone in serum is indirectly assayed by determining the available binding sites on thyroid binding globulin (TBG) present in the serum. In this test, it is assumed that the amount of TBG in normal sera is relatively constant and that it binds most of the available thyroid hormone. When labeled T-3 (triiodothyronine) is added to a serum sample, it will be bound by the TBG in proportion to the residual binding sites available thereon. Thus, if it is found that a large amount of labeled T-3 is bound by the serum, this indicates a large number of available binding sites and hence a low level of thyroid hormone, and *vice versa*. Measurement of the unbound labeled T-3 can thus be related to thyroid function. In the clinical application of the T-3 uptake test, it suffices in many cases to determine the T-3 uptake ratio in comparison with a standard normal serum. This ratio can be derived by dividing the partial count (as defined above) obtained from the unknown sample by the partial count of a standard serum sample which has been subjected to a parallel, identical assay procedure.

In a preferred embodiment, the determination of ligand binding capacity in the liquid medium, usually aqueous, is carried out by an improved radioassay method which comprises mixing the said aqueous medium with a radiolabeled form of the said ligand or of a binding analogue thereof and incubating the resulting mixture for a predetermined period of time to form a bound-species of the said radiolabeled ligand or analogue wherein such is bound to a binding agent from the said aqueous medium and a free-species of the said radiolabeled

ligand or analogue wherein such is not bound to the said binding agent, separating the said bound-species and the said free-species by contacting the said reaction mixture after the said predetermined incubation period, with a column comprising an adsorbent material which is selective for binding one of the said bound-species and free-species, capillarily absorbent relative to the said reaction mixture and of sufficient volume to be capable of drawing all of the said reaction mixture thereinto by capillary action, so that the said bound-species and free-species are separated along the said column, and measuring the radioactivity of one of the said separated species.

The present invention also provides a test kit for carrying out the present method. A test kit is provided for determining a ligand in a liquid medium, comprising (1) the said ligand, or a binding analogue thereof, incorporated with a label, such as a radioactive atom, (2) a binding agent for the said ligand, such as an antibody and (3) a column of an adsorbent material selective for one of the said ligand and the binding complex of the said ligand and the said binding agent, capillarily absorbent relative to the said liquid medium, and such as to be capable of drawing thereinto by capillary action all of an incubated reaction mixture formed by mixing the said liquid medium with the said labelled ligand a binding analogue thereof and the said binding agent and incubating the said mixture for a predetermined period of time, so that bound-species and free-species formed in the said incubated reaction mixture are separated along the said column. Also provided is a test kit for determining the ligand binding capacity of a liquid medium, comprising (1) said ligand, or a binding analogue thereof, incorporated with a label, such as a radioactive atom, and (2) a column of an adsorbent selective for one of the said ligand and the binding complex of the said ligand and a binding agent for said ligand suspected to be contained in the said medium, capillarily absorbent relative to the said liquid medium, and such as to be capable of drawing thereinto by capillary action all of an incubated reaction mixture formed by mixing the said liquid medium with the said labelled ligand a binding analogue thereof and incubating the said mixture for a predetermined period of time so that bound-species and free-species found in the said incubated reaction mixture are separated along the said column. The test kits may additionally comprise an aqueous buffer solution and ligand standards.

The present invention is illustrated by the following examples.

PREPARATION OF CROSS-LINKED POLYVINYL ALCOHOL

Hydrochloric acid (concentrated, 5 ml) was added to a stirred solution of 10 grams of cold water soluble crystalline Type II polyvinyl alcohol (PVA) (catalog No. P-8136, Sigma Chemical Co., St. Louis Missouri, U.S.A.) in water (600 ml). The resulting viscous solution was stirred with a high-powered stirrer and heated to 65°C. Glutaraldehyde (25% aqueous solution, 10 ml) was added and the reaction mixture was stirred at 65°C for 20 min. The resulting insoluble, cross-linked PVA was filtered and washed with distilled water until the pH of the washing was neutral. The wet cake was washed with ethanol or acetone and air-dried. The yield of the granular, white polymer was 11.0 g. No melting, softening or decomposition was observed on heating to 260°C. It was found insoluble in water and in organic solvents, including refluxing dimethylformamide (DMF).

PREPARATION OF ADSORBENT COLUMNS

Plastics tubes about 8 cm long and about 0.5 cm inner diameter were used. A porous retainer disc was pressed into the bottom of each tube in such a way that it would be flush with the bottom but would not fall out. The column was then evenly filled with about 0.1 g of a dry adsorbent (such as cross-linked polyvinyl alcohol prepared as above) to a height of about 6 cm and a second porous disc was pushed coaxially into firm contact with the top of the adsorbent bed.

EXAMPLE 1

Radioimmunoassay for Thyroxine (T-4)

In order to perform a radioimmunoassay for T-4, the following reagents, all dissolved in tris-maleate buffer pH 7.4 (prepared by dissolving 2.85 g tris-(hydroxymethyl)-aminomethane, 1.2 g maleic acid and 0.43 g ethylenediamine tetraacetic acid in 290 ml of distilled water) were added step-wise to test tubes:

1) 200 μl ^{125}I-T-4 (about 100 kcpm)
2) 200 μl T-4 standard (diluted 1:4 in concentration range of 4-60 μg/liter) or a 1:4 diluted clinical serum sample
3) 50 μl ANS solution (8-anilino-1-napthalene sulfonic acid, ammonium salt, 4 g/liter)
4) 200 μl anti-T-4 antibody (dissolved in 5 ml buffer).

The test tubes were apparently shaken after each of step 3 and step 4 to ensure thorough mixing

of the reagents. Then, following 20 minutes of incubation at room temperature, an adsorbent column, with cross-linked PVA as adsorbent, prepared as above, was placed vertically in the test tube and the reaction mixture allowed to ascend in the dry cross-linked polyvinyl alcohol. When all the reaction mixture had been adsorbed, the total radioactivity (total count) was determined. For this purpose the adsorbent column was introduced into the well of a gamma counter without any metal shield. The radioactivity of the intake portion of the column (holding free ^{125}I-T-4) was then determined (partial count) using the metal shield to prevent the radioactivity from the remaining part of the column from being counted. The percent retention of each column was calculated using the equation:

$$\text{percent retention} = \frac{\text{partial count}}{\text{total count}} \times 100$$

A standard curve was obtained by plotting the percent retention values versus the corresponding concentrations of thyroxine standard (Fig. 2).

Unknown amounts of T-4, e.g., in serum, can be determined in the above manner with the aid of the standard curve of Figure 2. Using this standard curve two reference sera identified as Led-I and Led-II obtained from Lederle Diagnostics, American Cyanamid Company, Pearl River, N.Y., U.S.A. were tested in duplicate.

Results were obtained as follows:

Led-I 7.0 and 7.9 μg%100 ml (expected value = 8-11 μg/100);
Led-II 16.5 and 17.6 μg/100 ml (expected value = 16.9-25.5 μg/100 ml).

EXAMPLE 2

Radioimmunoassay for Digoxin

Cross-linked polyvinyl alcohol adsorbent columns were prepared as described above. The buffer used in this test was a phosphate buffer, pH 7.4 (6.25 g/liter sodium dihydrogen phosphate, pH adjusted with sodium hydroxide solution).

In order to perform a radioimmunoassay for digoxin, the following reagents were added step-wise to test tubes:

1. 200 μl ^{125}I-digoxin (27 kcpm)
2. 150 μl standard digoxin. The standards were prepared by diluting 1 ml of stock solutions having concentrations of 0.5, 2.0 and 5.0 μl/ml with one ml of normal serum and one ml buffer. The standards were thus diluted 1:3.
3. 150 μl antidigoxin rabbit antiserum in phosphate buffer containing 0.2% bovine serum albumin.

The reaction mixture was incubated at room temperature for 40 minutes in the test tube. Then an adsorbent column as in Example 1 was placed vertically in the test tube and the reaction mixture allowed to ascend into the dry cross-linked polyvinyl alcohol. When all the reaction mixture had been adsorbed, total and partial radioactivity counts were performed and the percent retention calculated as in Example 1, and a standard curve was obtained in a similar manner (Fig. 3).

Unknown amounts of digoxin, e.g., in serum, can be determined in the above manner with the aid of the standard curve of Figure 3.

Using this standard curve the two reference sera (Led-I and Led-II) were tested in duplicate. Results were obtained as follows:

Led-I 1.0 ng/ml (expected value - 0.8-1.2 ng/ml).
Led II 3.8 ng/ml (expected value - 2.5-4.0 ng/ml).

EXAMPLE 3

Radioassay for Triiodothyronine (T-3) Uptake

Cross-linked polyvinyl alcohol adsorbent columns were prepared as described above. The buffer used in this test was made by dissolving citric acid (14.4 g) and sodium dihydrogen phosphate ($NaH_2PO_4.8H_2O$) (43.75 g) and 37% formaldehyde solution (2.7 ml) in distilled water (1 liter).

The test was performed by adding the following reagents to a test tube:

1. 200 μl ^{125}I-T-3 (in citric acid buffer, 120 kcpm)
2. 20 μl serum (standard or clinical sample).

The test tube was shaken gently to ensure thorough mixing of the contents. An adsorbent column as in Example 1 was then placed vertically in the test tube and the reaction mixture allowed to ascend in the cross-linked polyvinyl alcohol. Immediately after all the reaction mixture had been absorbed, 200 μl of buffer was added to the test tube and this was also allowed to ascend in the column.

A partial radioactivity count of the intake portion of the column was then performed using the

metal shield.

The result was calculated as follows:

$$\frac{\text{counts of clinical sample column}}{\text{counts of standard serum column}} = x$$

$x = 1$ was normal, $x < 1$ was low, $x > 1$ was high.

Results obtained for low, normal, and high sera in terms of known T-3 uptake values were as follows: 0.6, 0.9 and 1.5.

EXAMPLE 4

Radioimmunoassay for Thyroxine (T-4)

The procedure of Example 1 was repeated with the single difference that instead of the cross-linked PVA columns there were used columns of the same type filled with "Sephadex G-10" brand cross-linked dextran (manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden).

The percent retention was calculated as in Example 1 and the percent retention values obtained were plotted versus the corresponding concentrations of the T-4 standardss. The standard curve obtained is shown in Fig. 4.

Unknown amounts of T-4, e.g., in serum, were determined in the above manner with the aid of the standard curve.

EXAMPLE 5

Radioimmunoassay for Digoxin

The procedure of Example 2 was repeated replacing the cross-linked PVA columns with dextran columns as described in Example 4.

Total and partial radioactivity counts were performed and the percent retention calculated as described in Example 1. With the aid of the obtained standard curve shown in Fig. 5, unknown amounts of digoxin, e.g., in serum, were determined in the above manner.

EXAMPLE 6

Radioimmunoassay for Thyroxine (T-4)

A. Preparation of formaldehyde treated starch

To a suspension of 100 g of starch (Starch Soluble Analar brand, The British Drug House, Poole, England) in 200 ml of water, there were added 45 ml of a 40% aqueous formaldehyde solution, followed by 10 ml of concentrated hydrochloric acid. The mixture was stirred at room temperature for 18 hours and then filtered. The solid product was washed repeatedly with water until it was pH neutral, then washed with acetone and dried.

B. Preparation of adsorbent columns

The procedure for preparing the columns described above was followed, except that the columns were filled with formaldehyde treated starch, prepared as described in part A.

C. Radioimmunoassay for T-4

The following reagents, all dissolved in tris-maleate buffer were added step-wise to test tubes:

1) 100 μl ^{125}I-T-4 (approximately 90 kcpm).
2) 100 μl T-4 standard (diluted 1:4 in concentration range of 4-60 μg/liter) or a 1:4 diluted clinical serum sample.
3) 50 μl ANS solution (8-anilino-1-naphthalene sulfonic acid, ammonium salt, 4 g/l).
4) 100 μl anti-T-4 antibody (dissolved in 3 ml buffer).

The procedure of Example 1 was accurately repeated, and by plotting the calculated percent retention values the standard curve shown in Fig. 6 was obtained.

EXAMPLE 7

Radioimmunoassay for Digoxin

The procedure of Example 2 was followed, replacing the cross-linked PVA columns with columns filled with starch (about 0.5 g) (Starch Soluble Analar brand, The British Drug House, Poole, England).

The following volumes of reagents were used:

1) 150 μl ^{125}I-digoxin (27 kcpm).
2) 50 μl standard digoxin or serum sample.
3) 100 μl antidigoxin rabbit antiserum.

The standard curve obtained is shown in Fig. 7.

With the aid of the standard curve, two clinical reference sera were tested (triplicate assays) and the results compared with digoxin assays using standard methods. The following results

were obtained:
Serum sample No. 1 : 1.2 ng/ml (expected value 1.0-1.5 ng/ml)
Serum sample No. 2 : 3.7 ng/ml (expected value 2.0-5.0 ng/ml)

EXAMPLE 8

Radioimmunoassay for Digoxin

The procedure was similar to that of Example 2, substituting the cross-linked PVA columns with columns filled with silica gel (200-400 mesh, Kieselgel 60, Catalog No. 9385, E. Merck, Darmstadt, West Germany).

The following volumes of reagents were used:
1) 200 μl ^{125}I-digoxin.
2) 150 μl standard digoxin or serum sample.
3) 150 μl antidigoxin rabbit serum.

The reaction kixture was allowed to incubate at room temperature for 50 minutes, whereafter an adsorbent column was placed in the test tube and the reaction mixture was allowed to ascend into the dry silica gel. When all the reaction mixture had been adsorbed, a further volume of 400 μl of the phosphate buffer solution was added to the test tube and allowed to ascend into the silica gel column. Total and partial radioactivity counts were performed and partial retention values calculated as in Example 1. The standard curve obtained is shown in Fig. 8.

With the aid of the standard curve 20 replicate tests were performed on a pooled clinical serum. The following results were obtained:

Concentration of Digoxin

found : 2.9 ng/μl

determined by other test method*: 2.5–3.0 ng/μl

Coefficient of variance = 12%

* (Digoxin Test Kit, catalog no. 070–06, Schwarz-Mann. Orangeburg. New York, U.S.A.)

EXAMPLE 9

Radioassay for Triiodothyronine (T-3) Uptake

Glass Pasteur pipettes were cut so as to form a column about 7 cm long having an inner diameter of about 6 mm and a constriction at the bottom end. A small plug of glass wool was inserted down to the bottom of the column and the column was filled with fine silica gel (200-400 mesh). Kieselgel 60, catalog no.9385. E.Merck, Darmstadt, West Germany). A second plug of glass wool was inserted into the column in order to retain the contents in place.

The procedure, reagents and volumes were the same as in Example 3, except that after the reaction mixture had been adsorbed, 500 μl of distilled water (instead of the 200 μl of buffer in Example 3) were added to the test tube and allowed to ascend in the column.

The partial radioactivity count was performed and the T-3 uptake ratio (x) calculated as in Example 3. The results obtained for low, normal, and high sera in terms of known T-3 uptake value were as follows:

Serum:	*Standard*	*Low*	*Normal*	*High*
Counts:	26.9	19.3	27.3	45.9
T–3 uptake ratio calculated		0.72	1.02	1.71
T–3 uptake ratio determined by known method*		0.70	1.02	1.37

(Trilute brand T-3 Uptake Test Kit, Ames Company Division of Miles Laboratories Inc., Elkhart, Indiana, U.S.A.).

In our Appln.No.47958/77 (Serial No.1564579) we have described and claimed a specific binding assay method for determining a ligand in, or the ligand binding capacity of a liquid medium, which comprises combining the said liquid medium with assay reagent means including a binding component incorporated with a label to form a binding reaction system having a bound-species and a free-species of the said labeled component, separating the said bound-species and the said free-species of the said labeled component by selectively adsorbing said free-species of the labeled component onto a solid comprising a nonion-exchange cross-linked polyvinyl alcohol, and measuring the said label in one of the said separated bound-species and free-species of the labeled component; a test kit for determining a ligand in a liquid medium comprising (1) the said ligand, or a binding analog thereof, incorporated with a label, (2) a binding agent for said ligand, and (3) a solid nonion-exchange cross-linked polyvinyl alcohol; and a test kit for determining the ligand binding capacity of a liquid medium, comprising (1) the said ligand, or a binding analog thereof, incorporated with a label, and (2) a solid nonion-exchange cross-linked polyvinyl alcohol.

WHAT WE CLAIM IS:

1. A specific binding assay method for determining a ligand in, or the ligand binding capacity of, a liquid medium, which comprises, (A) when the said ligand is to be determined, combining the said liquid medium with an assay reagent means comprising (i) as labeled component, the said ligand or a binding analogue thereof, incorporated with a label and (ii) a binding agent for the said ligand; or when the ligand binding capacity of the said liquid medium suspected to contain a binding agent for the said ligand is to be determined, combining the said liquid medium with an assay reagent means comprising, as labeled component, the said ligand, or a binding analogue thereof, incorporated with a label, so as to form a binding reaction mixture having a bound-species as the said labeled component bound to the said binding agent and a free-species as the said labeled component not bound to the said binding agent; (B) separating the said bound-species and the said free-species by contacting at least a portion of the said binding reaction mixture a predetermined time after formation thereof with a column comprising an adsorbent material which is both selective for binding one of the said bound-species and free-species and capillarily absorbent relative to the said liquid medium so that the said portion of the binding reaction mixture is drawn into the said column by capillary action and the said bound-species and the said free-species are separated along the said column; and (C) measuring the said label in one of the separated species.

2. A method according to Claim 1 wherein after the said portion of the said reaction mixture has been drawn into the said column of adsorbent material, the same end of the said column as that contacted with the said reaction mixture is contacted with a volume of liquid inert with respect to the selective adsorption of the said one of the said bound-species and free-species, and the said inert liquid is drawn into the said column to enhance the separation of the said bound-species and free-species along the said column.

3. A method according to Claim 1 or 2 wherein the volume of the said adsorbent column is sufficiently large to allow capillary absorption of all of the said reaction mixture therein.

4. A method according to any of Claims 1 to 3, wherein the said adsorbent material is cross-linked polyvinyl alcohol, cross-linked dextran, starch, formaldehyde-treated starch, or silica gel.

5. A method according to any of Claims 1 to 4 wherein the said ligand to be determined is selected from antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites and pharmacological agents, and their receptors and binding substances.

6. A method according to any of Claims 1 to 4 wherein the said binding agent is an antibody.

7. A method according to any of Claims 1 to 6 wherein the said label is a gamma-emitting radioactive atom.

8. A radioimmunoassay method for determining a ligand in an aqueous medium, which comprises (A) mixing the said aqueous medium with a radiolabelled form of the said ligand or of a binding analogue thereof and with an antibody for said ligand and incubating the resulting reaction mixture for a predetermined period of time to form a bound-species of the said radiolabelled ligand or analogue wherein such is bound to the said antibody and a free-species of said radiolabelled ligand or analogue wherein such is not bound to the said antibody, (B) separating the said bound-species and the said free-species by contacting the said reaction mixture, after the said predetermined incubation period, with a column comprising an adsorbent material which is selective for binding one of the said bound-species and free-species, capillarily absorbent relative to the said reaction mixture, and of sufficient volume to be capable of drawing all of the said reaction mixture thereinto by capillary action,

so that the said bound-species and free-species are separated along the said column, and (C) measuring the radioactivity of one of the said separated species.

9. A method according to Claim 8 wherein after all of the said reaction mixture has been drawn into the said column of adsorbent material, the same end of the said column as that contacted with the said reaction mixture is contacted with a volume of liquid inert with respect to the selective adsorption of the said one of the said bound-species and free-species and the said inert liquid is drawn into the said column to enhance the separation of the said bound-species and free-species along the said column.

10. A method according to Claim 9 wherein the said inert liquid is water or an aqueous buffer solution.

11. A method according to any of Claims 8 to 10 wherein the measurement of the radioactivity of the one of the said bound-species and free-species is accomplished by placing the said column in a well of a radioactivity counting apparatus with that portion of the said column containing the other of the said bound-species and free-species being selectively shielded from the said counting apparatus by a radio-opaque material.

12. A method according to Claim 11 wherein the said shielded portion of the said column is shielded by use of a well-liner composed of radio-opaque material.

13. A method according to any of Claims 8 to 12 wherein the said adsorbent column is in the form of an elongated tube containing a volume of the said adsorbent material held in position by retaining means.

14. A method according to any of Claims 8 to 13 wherein the said adsorbent material is cross-linked polyvinyl alcohol.

15. A method according to any of Claims 8 to 13 wherein the said adsorbent material is cross-linked dextran.

16. A method according to any of Claims 8 to 13 wherein the said adsorbent material is formaldehyde-treated starch.

17. A method according to any of Claims 8 to 13 wherein the said adsorbent material is silica gel.

18. A method according to any of Claims 8 to 13 wherein the adsorbent material is starch.

19. A method according to any of Claims 8 to 18 wherein the said ligand to be determined is thyroxine.

20. A method according to any of Claims 8 to 18 wherein the said ligand to be determined is digoxin.

21. A radioassay method for determining the ligand binding capacity of an aqueous medium, which comprises mixing the said aqueous medium with a radiolabelled form of the said ligand or of a binding analogue thereof and incubating the resulting mixture for a predetermined period of time to form a bound-species of the said radiolabelled ligand or analogue wherein such is bound to a binding agent from the said aqueous medium and a free-species of the said radiolabelled ligand or analogue wherein such is not bound to the said binding agent, separating the said bound-species and the said free-species by contacting the said reaction mixture after the said predetermined incubation period with a column comprising an adsorbent material which is selective for binding one of the said bound-species and free-species, capillarily absorbent relative to the said reaction mixture, and of sufficient volume to be capable of drawing all of the said reaction mixture thereinto by capillary action, so that the said bound-species and free-species are separated along the said column, and measuring the radioactivity of one of the said separated species.

22. A method according to Claim 21 wherein after all of the said reaction mixture has been drawn into the said column of adsorbent material, the same end of the said column as that contacted with the said reaction mixture is contacted with a volume of liquid inert with respect to the selective adsorption of the said one of the said bound-species and free-species and the said inert liquid is drawn into the said column to enhance the separation of the said bound-species and free-species along the said column.

23. A method according to Claim 22 wherein the said inert liquid is water or an aqueous buffer solution.

24. A method according to any of Claims 21 to 23 wherein the measurement of the radioactivity of the one of the said bound-species and free-species is accomplished by placing the said column in a well of a radioactivity counting apparatus with that portion of the said column containing the other of the said bound-species and free-species being selectively shielded from the said counting apparatus by a radio-opaque material.

25. A method according to Claim 24 wherein the said shielded portion of the said column is shielded by use of a well-liner composed of radio-opague material.

26. A method according to any of Claims 21 to 25, wherein the said adsorbent column is in the form of an elongated tube containing a volume of the said adsorbent material held in position by retaining means.

27. A method according to any of Claims 21 to 26 wherein the said adsorbent material is

cross-linked polyvinyl alcohol.

28. A method according to any of Claims 21 to 26 wherein the said adsorbent material is cross-linked dextran.

29. A method according to any of Claims 21 to 26 wherein the said adsorbent material is formaldehyde-treated starch.

30. A method according to any of Claims 21 to 26 wherein the said adsorbent material is silica gel.

31. A method according to any of Claims 21 to 30 wherein the determined ligand binding capacity of the same aqueous medium is the capacity of the said medium to bind triiodothyronine.

32. A method according to Claim 8 substantially as described in any one of Examples 1, 2, 4, 5, 6, 7 or 8.

33. A method according to Claim 21 substantially as described in Example 3 or 9.

34. A test kit for determining a ligand in a liquid medium, comprising (1) the said ligand, or a binding analogue thereof, incorporated with a label; (2) a binding agent for the said ligand; and (3) a column of an adsorbent material selective for one of the said ligand and the binding complex of the said ligand and the said binding agent, capillarily absorbent relative to the said liquid medium, and such as to be capable of drawing thereinto by capillary action all of an incubated reaction mixture formed by mixing the said liquid medium with the said labelled ligand or binding analogue thereof and the said binding agent and incubating the said mixture for a predetermined period of time, so that bound-species and free-species formed in the said incubated reaction mixture are separated along the said column.

35. A test kit according to Claim 34 which additionally comprises an aqueous buffer solution and ligand standards.

36. A test kit according to Claim 34 or 35 wherein said adsorbent column is in the form of an elongated tube containing a volume of the said adsorbent material held in position by retaining means.

37. A test kit according to any of Claims 34 to 36 wherein the said adsorbent material is cross-linked polyvinyl alcohol, cross-linked dextran, starch, formaldehyde-treated starch, or silica gel.

38. A test kit according to any of Claims 34 to 37 wherein the said label is a radioactive atom.

39. A test kit according to any of Claims 34 to 38 wherein the said binding agent is an antibody.

40. A test kit for determining the ligand binding capacity of a liquid medium, comprising (1) the said ligand, or a binding analogue thereof, incorporated with a label, and (2) a column of an adsorbent material selective for one of the said ligand and the binding complex of the said ligand and a binding agent for said ligand suspected to be contained in the said medium, capillarily absorbent relative to the said liquid medium, and such as to be capable of drawing thereinto by capillary action all of an incubated reaction mixture formed by mixing the said liquid medium with the said labelled ligand or binding analogue thereof and incubating the said mixture for a predetermined period of time so that bound-species and free-species formed in the said incubated reaction mixture are separated along the said column.

41. A test kit according to Claim 40 which additionally comprises an aqueous buffer solution and a ligand binding capacity standard.

42. A test kit according to Claim 40 or 41 wherein the said adsorbent column is in the form of an elongated tube containing a volume of the said adsorbent material held in position by retaining means.

43. A test kit according to any of Claims 40 to 42 wherein the said adsorbent material is cross-linked polyvinyl alcohol, cross-linked dextran, starch, or silica gel.

44. A test kit according to any of Claims 40 to 43 wherein the said label is a radioactive atom.

45. The test kit of Claim 34 or 40 substantially as hereinbefore described with reference to Figure 1 of the accompanying drawings.

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4 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 1

Fig. 1

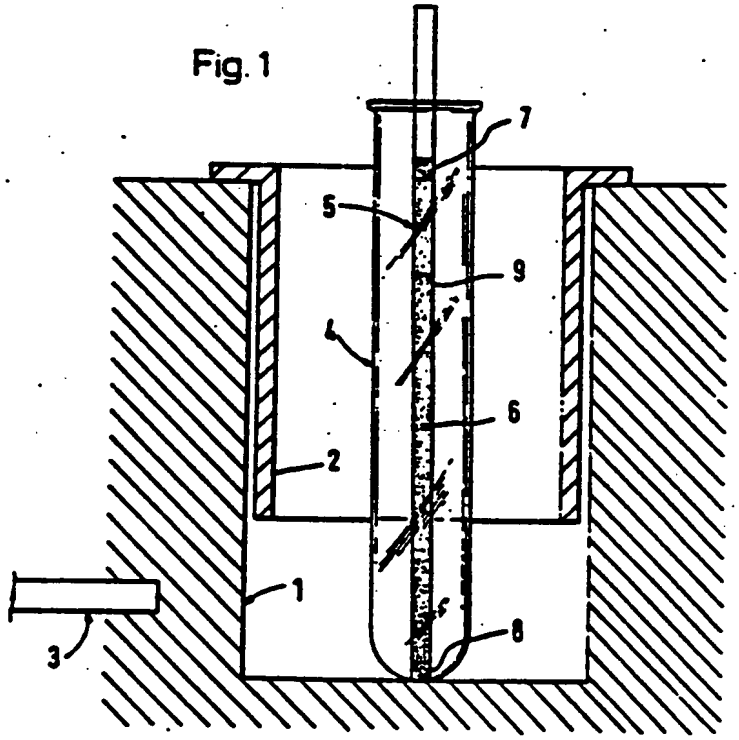
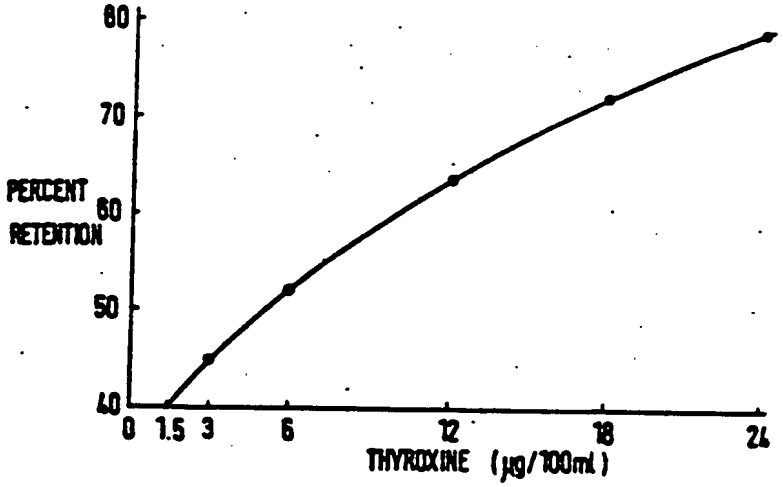
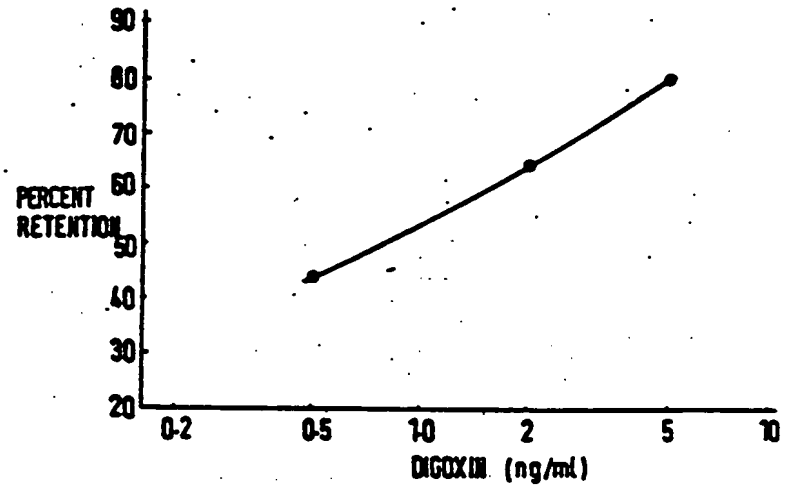


Fig. 2

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20
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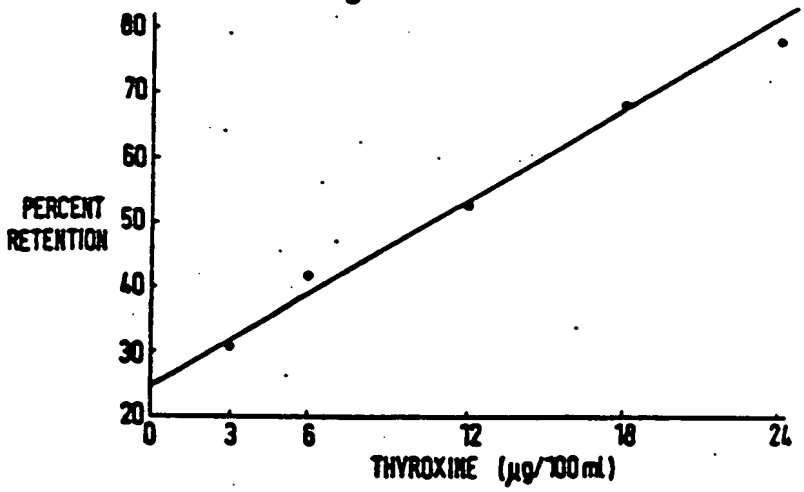
5
10
DIGOXIN (ng/ml)

Fig. 3

Fig. 4


80
70
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PERCENT RETENTION
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6
12
18
24
THYROXINE (µg/100 ml)

1564578 COMPLETE SPECIFICATION
4 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 3

Fig.5

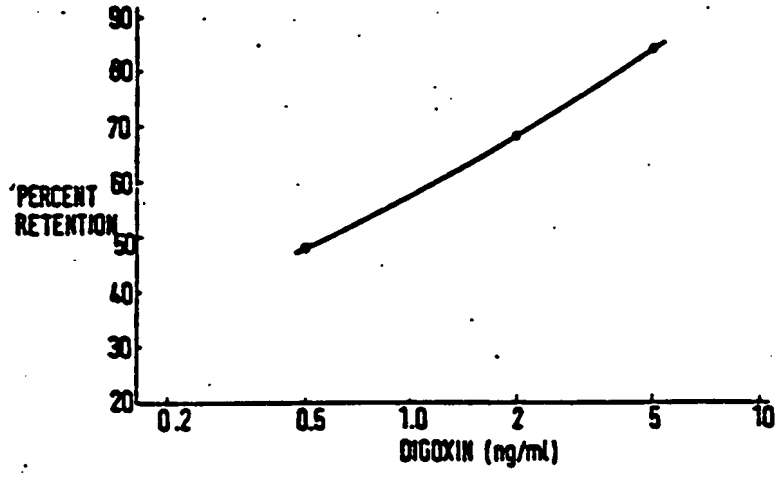
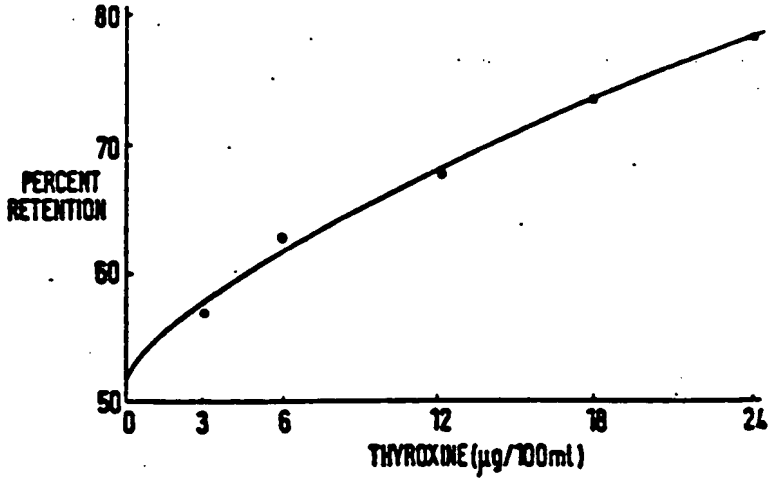
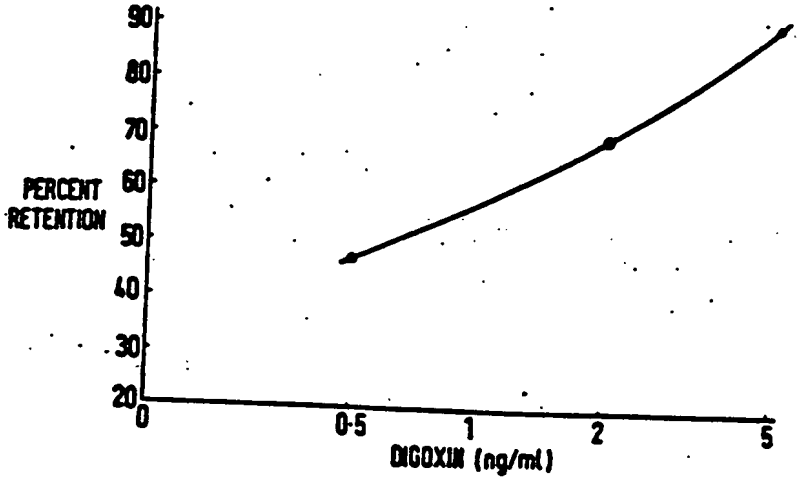


Fig. 6




PERCENT RETENTION
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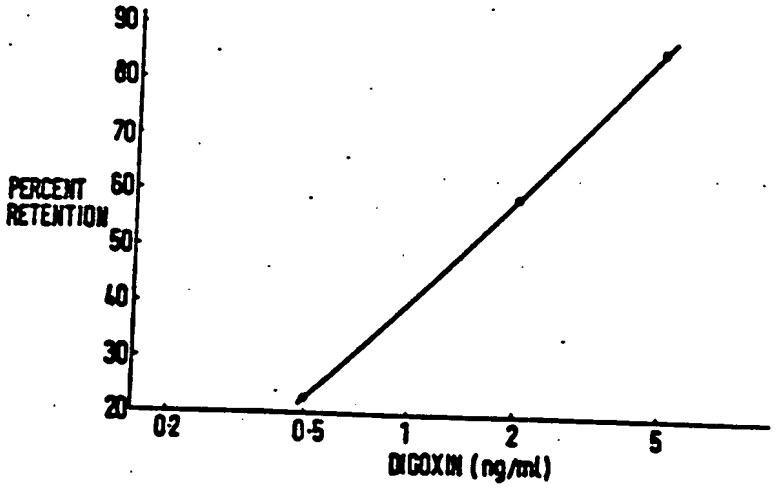
DIGOXIN (ng/ml)

Fig.7

Fig. 8


PERCENT RETENTION
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0·5
1
2
5
DIGOXIN (ng/ml)

DNA PROBES

George H. Keller
Mark M. Manak

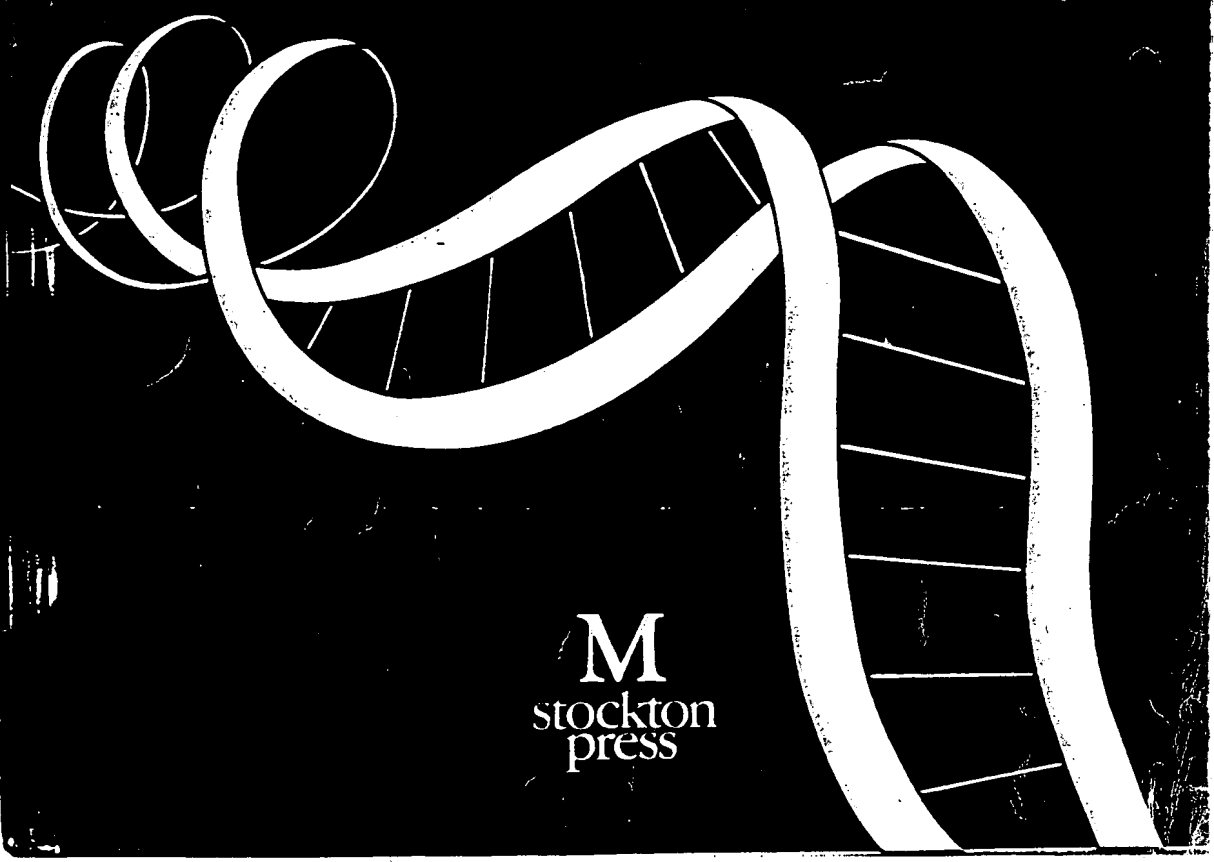

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Table 4.1 cont.

LABELING METHOD	DETECTION	REFERENCE	AFFILIATION	SENSITIVITY
CHEMICAL MODIFICATION				
oligonucleotides				
5'-amino	fluorescent primer	Smith *et al.* 1985	Cal Tech	NR
amino-cytosine	various groups	Ruth 1984	Molecular Biosystems	NR
amino-cytosine+ enzyme	direct	Jablonski *et al.* 1986	Molecular Biosystems	$3x10^6$ copies
5'-amino + enzyme	direct	Li *et al.* 1987	U. Adelaide	$3.5x10^6$ copies
		Sproat *et al.* 1987	EMBO	NR
SH-oligo + enzyme	direct	Chu and Orgel 1988	Salk Inst.	NR
Biotin-UMP	perox-Streptavidin	Cook *et al.* 1988	Enzo Biochem	$3x10^6$ copies
amino-cytosine+ enzyme	direct	Urdea *et al.* 1988	Chiron	$1.2x10^7$ copies

ENZYMATIC MODIFICATION

The first non-radioactive DNA probes, of a practical design, were described in the scientific literature by Langer *et al.* (1981). This early probe labeling scheme employed biotin-labeled deoxyribonucleotide triphosphates, incorporated into the probe DNA by enzymatic polymerization. The modified nucleotides, in turn, were developed as the result of years of experimentation with mercurated nucleotides and polynucleotides (Dale and Ward, 1975; Dale *et al.*, 1973; Dale *et al.*, 1975). The most widely used modified nucleotide is biotin-11-dUTP, as shown in Figure 4.1.

The molecule incorporates the following features: modification at the C-5 position where it will not interfere with hydrogen bonding, a double bond to minimize flexing of the linker arm and a linker arm long enough to ensure access of detection reagents to the biotin. This and other modified nucleotides can be incorporated into DNA by nick-translation (Leary *et al.*, 1983) or onto the ends of DNA by tailing (Riley *et al.*, 1986). After hybridization, these biotin-labeled probes are detected using avidin or streptavidin-enzyme conjugates. Streptavidin is superior to avidin for DNA detection because it exhibits far less nonspecific binding. Unlike avidin it contains no carbohydrate and has a neutral isoelectric point (Chaiet and Wolf, 1964). When combined with a precipitating substrate, the probe:immobilized target hybrid is visualized as a colored band or spot on nitrocellulose, (Leary *et al.*, 1983) or as cellular staining following *in situ* hybridization (Brigati *et al.*, 1983). These labeling and detection methods result in probes with a lower detection limit of 0.5-2 picograms, or about $5x10^4$ copies of target nucleic acid.

NUCLEIC ACID PROBES

Robert H. Symons

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I. INTRODUCTION

There is increasing interest worldwide in the development of nucleic acid probes which are detected by nonradioactive means. In the research laboratory, the use of ^{32}P for detection is undoubtedly the method of choice and is likely to remain so for the forseeable future, in spite of the half life of only 14 days for ^{32}P. In the diagnostic laboratory on the other hand, the use of nonradioactive probes has many potential advantages. Perhaps the major one is that nonradioactive probes are stable for at least 6 to 12 months, and probably much longer if properly stored, thus leading to a substantial reduction in cost by obviating the need to prepare them every 2 to 3 weeks. In addition, there is no radiation exposure from routine daily use and there are no storage and disposal problems.

The general field of nonradioactive probes is in a first generation stage where many different approaches are being investigated in the preparation of these probes and in the development of suitable detection methods. Very few systems using nonradioactive probes have reached the market place and there is unlikely to be a dramatic increase until the 1990s. There are a number of reasons for this. The well established ELISA procedures have a significant advantage in greater simplicity and their application to automated, quantitated print-out of data. They have found widespread application in diagnostic laboratories worldwide so that the use of nonradioactive probes must offer unique advantages to allow a competitive alternative. Perhaps the most important aspect is one of education, by demonstrating that probes can provide significant improvements in sensitivity and flexibility of design and application.

Numerous methods are described in this chapter for the preparation by enzymatic and chemical techniques of nonradioactive nucleic acid and oligonucleotide probes. In many cases, the resulting probes have yet to be fully tested under hybridization conditions. In others, initial results look very promising since some nonradioactive probes can provide a sensitivity of detection of target sequences similar to that provided by ^{32}P-labeled probes.

II. ENZYMATIC PREPARATION TECHNIQUES

A. Preparation of Nonradioactive, Labeled DNA

1. Incorporation of Biotinylated Nucleotides into DNA

a. Nick Translation Reaction

A landmark in the development of methods for labeling nucleic acid probes by nonisotopic means was the publication of Langer et al.[1] describing the synthesis of biotinylated nucleotides and their enzymatic incorporation into DNA and RNA. The biotin was attached via a linker arm to the 5-position of the pyrimidine ring of dUTP (Figure 1) or UTP. Such nucleotides could be incorporated by the respective polymerases into DNA or RNA. The length of the linker arm between the biotin and the pyrimidine ring was found to be important in the subsequent detection of biotinylated DNA when used as a probe; linker arms of either 11 or 16 atoms were better than those with only 4 atoms (Figure 1) for both *in situ*[2] and dot-blot[3] hybridizations.

Biotinylation of double-stranded DNA is readily achieved in a standard nick translation reaction (see Chapter 1, Section II.A) catalyzed by *E. coli* DNA polymerase I. For example, bio-11-dUTP is incorporated in place of dTTP into the DNA to the same extent as dTTP but at a slightly slower rate.[1] Using standard nick translation procedures, substitution of between 20 to 70% of the available deoxythymidine residues with biotinylated dUTP can be achieved. Reassociation kinetics of denatured normal and biotinylated double-stranded DNAs were identical,[1] indicating that the biotinylated DNA exists as an unperturbed double-helix. Hence, established hybridization procedures can be used with biotinylated DNA probes prepared in this way.

In addition to the biotinylated dUTP analogues (Figure 1), a series of biotinylated dATP and dCTP analogues have been prepared which were incorporated into DNA probes by nick translation.[4] Bio-7-dATP and bio-7-dCTP (Figure 2) gave the highest incorporation in the series of analogues where N (the number of atoms between the carbonyl group of the biotin moiety and the amino group of adenine or cytosine) varied from 3 to 17. All the results obtained with DNA probes labeled with either bio-7-dATP or bio-7-dCTP were very similar to those obtained with bio-11-dUTP.[2,3] Hence, none of these biotinylated probes offers any advantages over the latter.

b. Replacement Synthesis Using T4 DNA Polymerase

As considered in Chapter 1 (Section II.D), T4 DNA polymerase can be used to generate high specific activity hybridization probes from double-stranded DNA. The same reaction can be used to incorporate bio-11-dUTP into DNA fragments with almost the same efficiency as the incorporation of dTTP.[5] The resulting biotinylated DNA probe was successfully used in colony hybridization.

c. Use of Other DNA Polymerases

Bio-dUTP is not a suitable substrate for the avian myeloblastosis virus (AMV) reverse transcriptase[1,6] in the preparation of long cDNA copies of RNA. However, the recently cloned form of the reverse transcriptase of Moloney murine leukemia virus[7,8] does incorporate bio-11-dUTP into full length cDNA, although somewhat less efficiently (35%) as compared with dTTP.[6]

activity according to the method of Goldman and Lenhoff.[18] The immobilized NADP completely adsorbs the enzyme. The agarose pellet is suspended in 0.5 ml of 10 m*M* Tris chloride at pH 8.1 containing 4 m*M* NADP and is stirred for 5–10 minutes and centrifuged. The supernatant fluid is checked for dehydrogenase activity. The enzyme is readily recovered by this treatment.

Adsorption and Elution of Heavy Meromyosin (HMM) from Agarose-ATP Resin. To a suspension of 3 ml containing 1 ml of Sepharose-ATP and Ca^{2+} (2 m*M*) in 10 m*M* imidazole buffer at pH 7, 1.6 mg of heavy meromyosin are added. After 15 minutes of stirring at 0°, the suspension is centrifuged, and the ATPase activity and protein concentration are determined in the supernatant liquid. The active heavy meromyosin is almost completely adsorbed by the ATP-agarose conjugate. Mixtures containing water or Sepharose hydrazide instead of Sepharose-ATP are used as controls. To elute the HMM, concentrated solutions of ATP or ADP are added to give a final concentration of 5 m*M* nucleotide. After 15 minutes of stirring at 0°, the ATPase activity and the protein concentration of the supernatant are measured. Most of the adsorbed HMM is released by this treatment.

[18] R. Goldman and H. M. Lenhoff, *Biochim. Biophys. Acta* 242, 514 (1971).

[56] Nucleoside Phosphates Attached to Agarose

By ROBERT BARKER, IAN P. TRAYER, and ROBERT L. HILL

Nucleoside mono-, di-, and triphosphates and many of their derivatives, such as the sugar nucleotides and the vitamin-containing coenzymes, are substrates, inhibitors, or cofactors for a wide variety of enzymes. Thus, nucleotide phosphates attached to agarose should have wide application as affinity adsorbents for the purification of many different enzymes.

Derivatives of nucleoside phosphates containing a primary amino group can be coupled readily to cyanogen bromide-activated agarose. The major problem in preparation of the adsorbents is the design and synthesis of the nucleotide ligands to be attached to agarose. At present, four kinds of ligand (1, 2, 3, and 4) have been synthesized containing an alkyl- or aryl-amino group attached to either the phosphate, the purine base, or the ribosyl ring of the nucleotide.

Compound (1), 3′-(4-aminophenylphosphoryl)deoxythymidine 5′-phosphate, is a good inhibitor of staphylococcal nuclease and reacts with agarose through the *p*-amino group to give a useful adsorbent for the

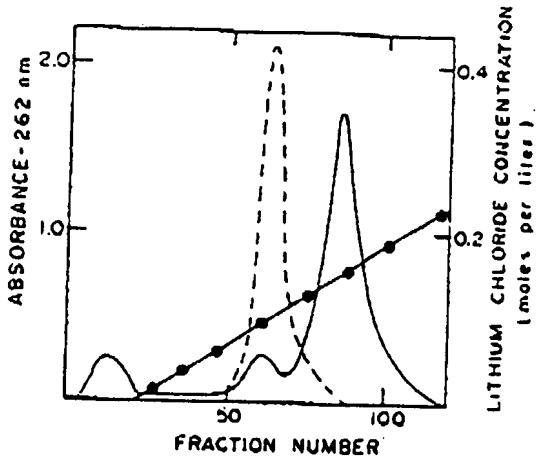


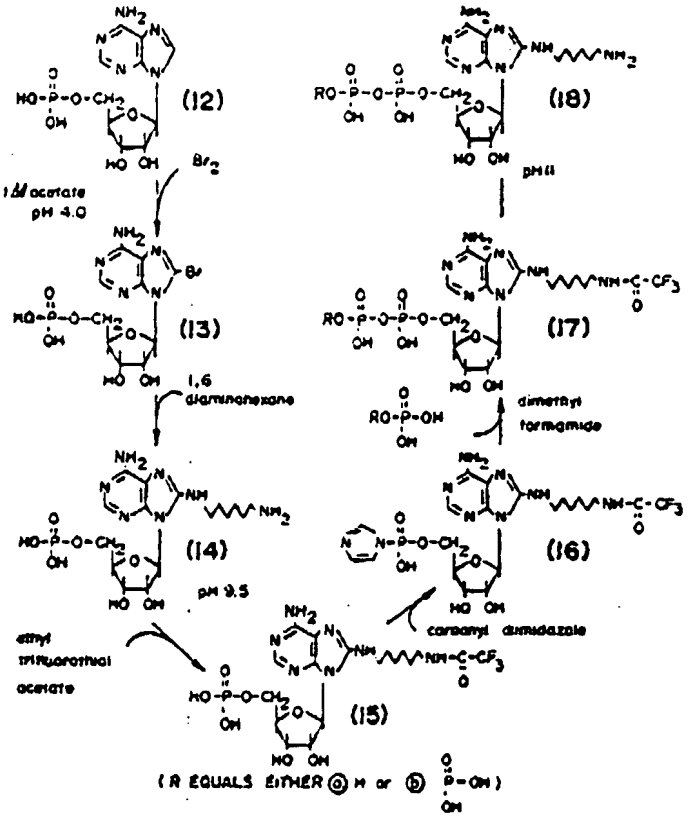
FIG. 1. The purification of trifluoroacetylhexanolamine-UDP and UDP-hexanolamine by chromatography on Dowex 1 (Cl-cycle) [R. Barker, K. W. Olsen, J. H. Shaper, and R. L. Hill, *J. Biol. Chem.* 247, 7135 (1972)]. Trifluoroacetylhexanolamine-UDP, the reaction mixture from 1 mmole of UMP was applied to a column (1 × 25 cm) of Dowex 1-X2 (Cl-cycle; 200–400 mesh) and eluted with 250 ml of 50% aqueous methanol and then with a linear gradient of 500 ml of 0.01 *N* HCl as starting solvent and 500 ml of 10 m*M* HCl containing 0.4 *M* lithium chloride as the limit solvent. Fractions (12 ml) were collected automatically. The column was operated at 25° at a flow rate of about 60 ml per hour. The elution pattern is shown by the solid line. UDP-hexanolamine-UDP, which was prepared as described in the text, was chromatographed exactly as described above. The elution pattern is shown by the dashed line.

by reaction of the ester, e.g., compound 2, with cyanogen bromide-treated Sepharose. Activation of the Sepharose and the coupling procedures used are essentially identical to that in this series [29, Section D]. The extent of coupling can be estimated by measuring the decrease in adsorbance during coupling or by phosphate analysis. An example of the use of UDP-hexanolamine-Sepharose for enzyme purification is the purification of a galactosyl transferase from bovine whey.[2]

B. *Adenosine Phosphate Ligands Substituted in the Adenine Ring*

The synthesis of N^6-(6-aminohexyl)-adenosine 5′-phosphate is given in this volume (Mosbach [16] and Deon and Harvey [17]). This section is devoted to the 6-aminohexyl derivative of 8-aminoadenine nucleotides. The synthetic sequence used is shown in Scheme II. In principle, the monobromo-derivatives of other nucleosides[21-23] could be treated in an

[21] R. E. Holmes and R. K. Robins, *J. Amer. Chem. Soc.* 86, 1242 (1964).
[22] A. M. Michelson, J. Dondon, and M. Grunberg-Manago, *Biochim. Biophys. Acta* 55, 529 (1962).
[23] D. M. Frisch and D. W. Visser, *J. Amer. Chem. Soc.* 81, 1756 (1959).



SCHEME II

analogous manner to provide a range of mononucleoside or mononucleotide derivatives suitable for affinity chromatography. Reaction of nicotinamide mononucleotide with the above imidazolide (16, Scheme II) should give an NAD^+ ligand which could be coupled to agarose through C-8 of the adenine ring. In addition, the N^6-(6-aminohexyl)-adenosine 5′-monophosphate derivative has been converted to the corresponding ADP and ATP derivatives by the imidazolide methods described below for the 8-amino derivative.

8-Bromoadenosine 5′-Monophosphate[21] (13). Disodium adenosine-5′-monophosphate (0.9 g, 2 mmoles) is dissolved in 1 *M* sodium acetate at pH 4.0 (80 ml), and bromine–water (0.15 ml bromine, 3 mmoles, dissolved in 15 ml of water with vigorous shaking) is added. After thorough mixing, the reaction mixture is stored in the dark at room temperature for 18 hours. The color of the solution is discharged by the addition of sodium metabisulfite (0.1 g) and the solvent is removed under reduced pressure.[16] The residue is concentrated several times from ethanol to give a white solid which is dissolved in 100 ml of water. The solution is added

[21] M. Ikehara and S. Uesugi, *Chem. Pharm. Bull.* 17, 348 (1969).

meeting one of the observations reported was the ability of EC cells, transplanted into a blastocyst, to participate in normal embryonic development, leading to a chimaeric mouse with tissues derived from both the host blastocyst and the EC cell[11–13]. However, the efficiency of chimaerism with most EC cell lines is low; this may be a result of small chromosomal abnormalities of many EC cells after prolonged *in vitro* culture, although other factors may be involved since some apparently euploid lines also fail to form chimaeras (J. Rossant and M. McBurney, Brock University, Ontario). Since the embryo-derived EC cells seem to have less chromosomal abnormalities, at least in early passage, the hope is that these may produce much higher rates of chimaerism.

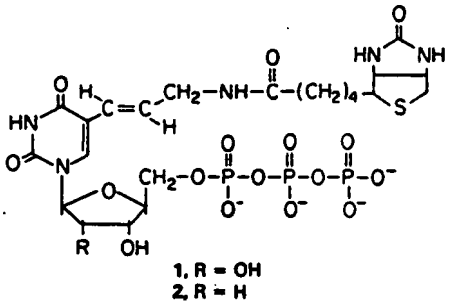
The other important possibility stemming from this technical advance is that of readily introducing known embryonic mutant genes into EC lines *in vitro*. For example, embryos homozygous for mutants of the *T/t* complex die at various stages of development. The mechanism of action of these lethal genes remains obscure, but suggestions varying from the aberrant expression of cell-surface molecules important in critical cell interactions to general metabolic deficiencies have been proposed. An embryo-derived EC cell line, homozygous for t^{w5}, which causes embryonic death at about day 8 of development, has been isolated (T. Magnusson, UCSF). However, this t^{w5}/t^{w5} EC cell line grows normally *in vitro* and forms well differentiated tumours when injected into mice. These observations suggest that embryonic lethality caused by this mutant is due to inability to organize embryonic structures at the proper time, rather than an inability simply to grow and differentiate. □

Lighting up time

from Sue Malcolm

THE report from David Ward's laboratory at Yale University School of Medicine of a new method for detecting specific DNA sequences suggests that a considerable increase in both sensitivity of detection and resolution may be just round the corner. The technique may be particularly valuable in the antenatal detection of those hereditary diseases where the molecular and chromosomal mapping of the genes involved has already been made possible. The basic idea is to incorporate a biotin-labelled nucleotide into the nucleic acid probe, to bind the substituted nucleic acid with an anti-biotin antibody (raised in rabbit) and then detect the complex either by fluorescence with a second antibody (goat anti-rabbit) coupled to fluorescein or cytochemically with the second antibody coupled to an enzyme such as horseradish peroxidase.

Present techniques rely on radiolabelling cloned DNA fragments with ^{32}P- or ^{125}I-labelled nucleotides. After hybridization, the labelled sequences can be detected with great sensitivity, but there are a number of limitations to the use of the isotopes. ^{32}P has a half-life of only two weeks and probes must be made and used immediately. This presents particular difficulties for laboratories any distance from the major suppliers (such as clinical laboratories in many of the countries where antenatal diagnosis of the haemoglobinopathies is desirable), and the handling and disposal of the radio-isotopes presents a health hazard, not to mention a bureaucratic hazard. The high energy of the radioactive emissions, particularly of ^{32}P, makes the grains on the film too scattered to give useful resolution in detecting specific mRNA sequences in individual cells, or genes on chromosomes. Tritiated nucleotides give much better resolution in cytological specimens but are necessarily also of lower specific activity, and the amount of nucleic acid corresponding to a single-copy gene on an individual chromosome or a low-abundance mRNA in a single cell is so low that their detection only becomes possible with the use of very long autoradiographic exposure times or the pooling of data.



Biotin-labelled derivatives of UTP (1) and dUTP (2). In 1, R = OH and in 2, R = H.

The new technique makes use of biotin-labelled derivatives of dUTP and UTP (see the figure) synthesized via 5-mercurated intermediates (Langer, Waldrop & Ward *Proc. natn. Acad. Sci. U.S.A.* 78, 6633; 1981). Other nucleotides could be synthesized by the same method but dUTP and UTP have proved the easiest to make. The biotin derivatives can be introduced easily into RNA and DNA. Bio-dUTP is a good substrate for the enzymes DNA polymerase I of *Escherichia coli* and bacteriophage T4 DNA polymerase and can be incorporated efficiently into DNA by 'nick-translation'. Bio-UTP can be incorporated into cRNA by *E. coli* RNA polymerase. Unfortunately, the analogues do not seem to function as substrates for avian myeloblastosis virus reverse transcriptase or for eukaryotic RNA polymerases.

In hybridization, Bio-substituted nucleic acids behave similarly to the non-substituted versions. There is no change in the melting temperature of DNA containing 20 biotin molecules per kilobase introduced by nick translation. Viral DNA in which all the thymidines in one strand are replaced by Bio-dUMP shows a drop in melting temperature of only 5°C. Lightly substituted DNA (5.5 per cent of thymidine substituted by Bio-dUMP) hybridizes in solution at the same rate as non-substituted DNA. The substitution may have some useful side effects — the hydrophobic side chain added to the DNA changes its physical properties and, for example, results in it being extracted into the phenol phase during phenol extraction. This could cut down background during hybridization on either nitrocellulose filters or on glass-mounted cytological preparations.

Langer-Safer and colleagues (Langer-Safer, Levine & Ward *Proc. natn. Acad. Sci. U.S.A.* 79, 4381; 1982) have now used the biotin-labelled DNA to detect specific sequences in *Drosophila* polytene chromosomes by *in situ* hybridization. Here there are no problems of detection as the DNA in the polytene chromosomes is replicated many times. The probes were prepared with between 8 per cent and 40 per cent of their thymidine residues substituted with Bio-dUMP, and after hybridization to the chromosomes they were reacted with rabbit anti-biotin antibody. For direct fluorescence detection the slides were incubated with goat anti-rabbit antibody labelled with fluorescein and examined by phase-contrast and fluorescence microscopy. There are a few disadvantages to this method, in particular bleaching of the fluorescent signal under the light source, so a second method, using a histochemical stain, was also employed. The slides were incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG and the peroxidase activity then used to convert the substrate diaminobenzidine into an insoluble brown precipitate which can be clearly distinguished in Giemsa-stained preparations. One valuable aspect of this method is that the length of chemical exposure can be continuously monitored to give an optimal reaction time. The same method has been used by Manuelidis to detect repeated sequences in human chromosomes and mouse satellite sequences in mouse chromosomes [in *Genome Evolution* (eds Dover, G.A. &

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Flavell, R.B.) 263, Academic, New York; 1982].

The method has many potential variations, the most obvious being the use of avidin-bound enzyme complexes (to exploit the biotin/avidin K_d of 10^{-15}) or the use of biotin derivatives with longer linker arms (to increase accessibility). In fact a derivative, Bio II-dUTP, with an extra seven atoms in the linker arm, is already commercially available. The day may not be far removed when you carry your Southern blot filters to lunch with you and check their progress every now and again. □

More to melanism than meets the eye

from J.S. Jones

INDUSTRIAL melanism is the textbook example of natural selection in action. Like most such examples it is usually presented in a way which is both incomplete and inaccurate. The popular view is that a mutation to a black form appeared in the previously light-coloured populations of the peppered moth *Biston betularia* at the beginning of the Industrial Revolution. As in a sooty environment the melanics were well concealed from predators they replaced non-melanics in polluted cities while remaining at a low frequency in the countryside. The real story is, however, much more complex and a recent computer model, produced by G.S. Mani, a University of Manchester physicist, shows that selective predation, differences in fitness unrelated to crypsis and gene flow must all be taken into account before many puzzling features of melanism can be explained.

Melanism has always presented a number of problems. It appeared in industrial Britain not only in *Biston* and more than a hundred other moths, but in species not subject to intensive predation such as beetles, cats and birds[1]. In ladybirds, melanics became more common mainly because the increased efficiency of absorption of solar energy gives them an advantage in the smoky atmosphere of cities[2]. Melanic pigeons are favoured in towns because the associated hormonal changes make them better able to exploit the possibilities for winter breeding presented by the year-round availability of food[3]. In both these distantly related organisms, the mating advantage of melanic males over typicals may also be important in their evolution[4]. Although in the peppered moth the first melanic was not recorded until 1848 in Manchester, dark forms of other moths were common in forests long before the industrial revolution, and in the newly polluted cities of north-east America, industrial melanism evolved exceptionally rapidly as these variants spread from the forests. Even the apparently simple case of industrial melanism in *B. betularia* involves at least four alleles, and many British populations contain three phenotypically distinguishable classes — the pale form *typica*, the intermediate *insularia* and the full melanic *carbonaria*[1,5].

There are several puzzling aspects of melanism in *Biston*. Even in the most polluted areas, such as central Manchester, the frequency of melanics never rose above 95 per cent, although field experiments on marked individuals show that melanics are considerably better camouflaged and should have replaced lighter forms long ago if only differential predation is involved[6]. When pollution was reduced the frequency of *carbonaria* fell rapidly (from 95 per cent to 82 per cent between 1960 and 1975 near Liverpool) although the melanics still appeared better camouflaged than typicals. In addition, melanic alleles in *B. betularia* reach a frequency of 80 per cent in rural East Anglia, where there is little industrial pollution[7]. None of these observations can be explained by simple directional selection by predators.

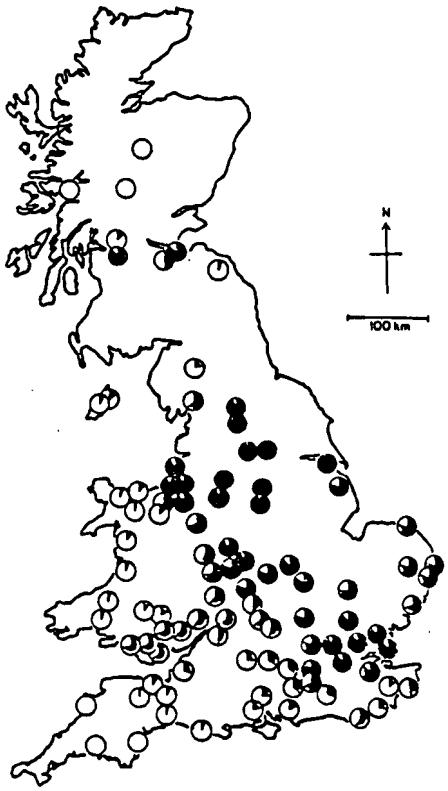

Fig. 1 Distribution of melanics in *Biston betularia*. White sector: *typica*; black sector: *carbonaria*; halved sector: *insularia* (from ref. 1).

Some of the apparent anomalies in the distribution of melanic *B. betularia* are related to its powers of migration. Males migrate about 2.5 km per generation, and this results in considerable gene flow between polluted and unpolluted places[8]. Computer simulations show that a balance between selection and migration is enough to explain the persistence of typicals in highly polluted areas[6], and partly explains why the rapid decrease in melanic frequency does not coincide with the urban boundaries of Liverpool and Manchester. However, gene flow alone cannot explain the position of this cline, nor why melanics are so common in some unpolluted parts of Britain[9].

Mani's model incorporates new information on fitness differences between melanics and typicals which are unrelated to visual selection by predators[10]. Breeding experiments involving 12,569 larvae from 83 families show such that differences are quite considerable; for example, *typica* homozygotes are at an approximately 30 per cent disadvantage to *carbonaria* homozygotes and a 7 per cent disadvantage to *insularia* homozygotes when raised in the laboratory[11]. There is no evidence of heterozygote advantage although this has often been proposed as a mechanism for maintaining melanic polymorphism. In the model, England and Wales are divided into squares with 22.5 km sides; mutation takes place at a rate of 10^{-6} per generation, and pollution in cities is assumed to have increased linearly for 55 years from 1830 and since then to have remained constant. The simulation, which incorporates experimental data on differential selection by predators, gene flow and spatially constant non-visual differences in fitness, was allowed to proceed for the 150 *B. betularia* generations from the beginning of the Industrial Revolution to the present day. It generates a distribution of melanics which is remarkably close to that found in modern populations, with an incomplete replacement of typicals even in the most polluted cities, and local patches of high melanic frequency in some unpolluted regions.

The best fit of Mani's predicted frequencies to those found in nature is obtained using estimates of non-visual

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1. Lees, D.R. in *Genetic Consequences of Man Made Change* (eds Bishop, J.A. & Cook, L.M.) (Academic, New York, 1981).
2. Muggleton, J. *Heredity* 40, 269 (1978).
3. Murton, R.K. *et al. J. Reprod. Fert.* (Suppl.) 19, 561 (1977).
4. Majerus, M. *et al. Heredity* 49, 37 (1982).
5. Lees, D.R. & Creed, E.R. *Heredity* 39, 67 (1977).
6. Cook, L.M. & Mani, G.S. *Biol. J. Linn. Soc.* 13, 179 (1980).
7. Bishop, J.A. & Cook, L.M. *Adv. Ecol. Res.* 11, 373 (1980).
8. Bishop, J.A. *J. Anim. Ecol.* 41, 209 (1972).
9. Mani, G.S. *Proc. R. Soc.* B210, 299 (1980).
10. Mani, G.S. *Biol. J. Linn. Soc.* 17, 259 (1982).
11. Creed, E.R. *et al. Biol. J. Linn. Soc.* 13, 251 (1980).

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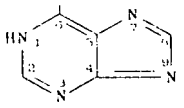


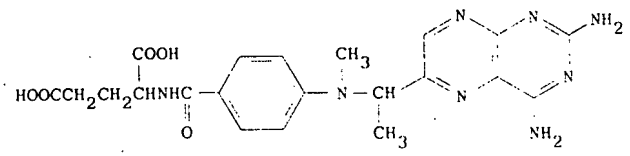
Trihydrate, orthorhombic needles. Anhydr at 110°, dec 360-365°, subl 220°. uv max (pH 7.0): 207, 260.5 nm ($\epsilon \times 10^{-3}$ 23.2, 13.4). One gram of anhydr compd dissolves in 2000 ml water, 40 ml boiling water; slightly sol in alc; practically insol in ether, $CHCl_3$. Aq solns are neutral. Combines with acids and bases. LD_{50} orally in rats: 745 mg/kg, Philips *et al., J. Pharmacol. Exp. Ther.* **104,** 20 (1952).

Hydrochloride hemihydrate, monoclinic prisms. One gram dissolves in 42 ml water.

Sulfate dihydrate, crystals. One gram dissolves in 150 ml water; slightly sol in alc.

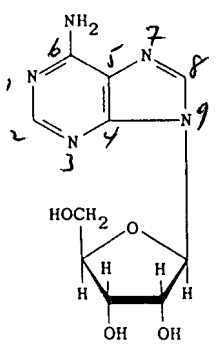
USE: In microbial determination of niacin; in research on heredity, virus diseases, and cancer.

143. A-Denopterin. *N-[4-[[1-(2,4-Diamino-6-pteridinyl)ethyl]methylamino]benzoyl]-L-glutamic acid;* 4-amino-N^{10},9-dimethylpteroylglutamic acid. $C_{21}H_{24}N_8O_5$; mol wt 468.48. C 53.84%, H 5.16%, N 23.92%, O 17.08%. Prepn: Hultquist *et al., J. Am. Chem. Soc.* **71,** 619 (1949); Hultquist, Smith, **Brit. pat. 667,098** (1952 to Am. Cyanamid).



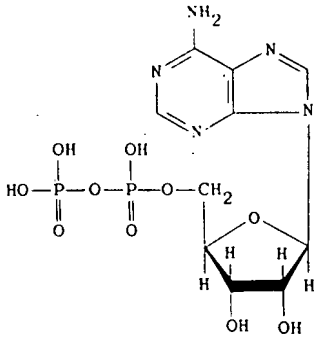
Dihydrate, yellow-orange microcrystals. uv max (0.1 *N* NaOH): 255, 306, 369 nm (ϵ 25,000, 25,000, 9000); (0.1 *N* HCl): 244, 316 nm (ϵ 22,000, 14,000).

144. Adenosine. *9-β-D-Ribofuranosyl-9H-purin-6-amine;* 6-amino-9-β-D-ribofuranosyl-9*H*-purine; 9-β-D-ribofuranosidoadenine; adenine riboside. $C_{10}H_{13}N_5O_4$; mol wt 267.24. C 44.94%, H 4.90%, N 26.21%, O 23.95%. Nucleoside; widely distributed in nature. From yeast nucleic acid: Levene and Bass, *Nucleic Acids* (New York, 1931) p 163. Structure: Levene, Tipson, *J. Biol. Chem.* **94,** 809 (1932); Bredereck, *Ber.* **66,** 198 (1933); *Z. Physiol. Chem.* **223,** 61 (1934); Gulland, Holiday, *J. Chem. Soc.* **1936,** 765. *Cf.* Szent-Györgyi, *J. Physiol.* **68,** 213 (1930); Lythgoe *et al., J. Chem. Soc.* **1947,** 355; **1948,** 965. Synthesis: Davoll *et al., ibid.* **1948,** 967; H. Vorbrueggen, K. Krolikiewicz, *Angew. Chem. Intl. Ed.* **14,** 421 (1975). Crystal structure: T. F. Lai, R. E. Marsh, *Acta Crystallogr.* **B28,** 1982 (1972). Conformational properties: D. B. Davies, A. Rabczenko, *J. Chem. Soc. Perkin Trans. 2* **1975,** 1703. *Reviews: see* Adenine; Nucleic Acids.



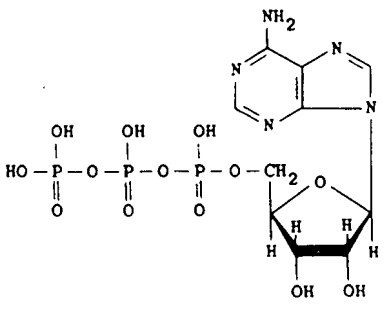
Crystals from water, mp 234-235°. $[\alpha]_D^{11}$ −61.7° (c = 0.706 in water); $[\alpha]_D^{9}$ −58.2° (c = 0.658 in water). uv max: 260 nm (ϵ 15,100). Practically insol in alcohol.

145. Adenosine Diphosphate. *Adenosine 5'-(trihydrogen diphosphate);* ADP; adenosine 5'-pyrophosphoric acid; 5'-adenylphosphoric acid; adenosinediphosphoric acid. $C_{10}H_{15}N_5O_{10}P_2$; mol wt 427.22. C 28.11%, H 3.54%, N 16.39%, O 37.45%, P 14.50%. Formed from ATP in the muscle by the enzyme adenosinetriphosphatase upon stimulation of the muscle unless hydrolysis is prevented by injection of magnesium sulfate. Prepd by hydrolysis of ATP by means of adenosinetriphosphatase from lobster or rabbit muscle: LePage, *Biochem. Prepns.* **I,** 1 (1949). Synthesis: Chambers *et al., J. Am. Chem. Soc.* **82,** 970 (1960). *See also* the ref and data *under* Adenosine Triphosphate.



Barium salt, $Ba_3(C_{10}H_{12}N_5O_{10}P_2)_2$. The purity of the prepns can be checked by analyses for nitrogen, ribose (orcinol reaction), total phosphorus, easily hydrolyzable phosphorus, and inorganic phosphorus. ADP should give a ratio of 2:1 for total organic phosphorus to easily hydrolyzable phosphorus, *see* LePage, *loc. cit.* a_M (molar absorbancy of pure ADP): 15.4 × 10^3 at 259 nm and pH 7.0.

146. Adenosine Triphosphate. *Adenosine 5'-(tetrahydrogen triphosphate);* ATP; adenosine 5'-triphosphoric acid; Adephos; Adetol; Atipi; Atriphos; Striadyne; Triadenyl; Triphosaden. $C_{10}H_{16}N_5O_{13}P_3$; mol wt 507.21. C 23.68%, H 3.18%, N 13.81%, O 41.01%, P 18.32%. Coenzyme valuable in the transfer of phosphate bond energy. Mammalian skeletal muscle at rest contains 350-400 mg ATP per 100 g. Upon stimulation of the muscle the ATP is hydrolyzed to ADP by the myosin-actin complex unless hydrolysis is prevented by injection of magnesium sulfate. Isoln from rabbit muscle: LePage, *Biochem. Prepn.* **1,** 5 (1949); *cf.* Fiske, Subbarow, *Science* **70,** 381 (1929); Lohmann, *Biochem. Z.* **223,** 460 (1931); **254,** 381 (1932); Barrenscheen, Filz, *ibid* **250,** 281 (1932); Kerr, *J. Biol. Chem.* **139,** 121 (1941); Needham, *Biochem. J.* **36,** 113 (1942). Synthetic routes: Tanaka, Honjo, U.S. pat. **3,079,379** (1963 to Takeda). Reviews of biosynthesis: Racker, *Advan. Enzymol.* **23,** 323-399 (1961); Deamer, *J. Chem. Ed.* **46,** 198-206 (1971). Reviews of nucleotide coenzymes: Bock in *The Enzymes* **vol. 2A,** P. D. Boyer *et al.,* Eds. (Academic Press, New York, 2nd ed., 1960) pp 3-38; A. M. Michelson, *The Chemistry of Nucleosides and Nucleotides* (Academic Press, New York, 1963) pp 153-250; D. W. Hutchinson, *Nucleotides and Coenzymes* (John Wiley, New York, 1964) pp 36-82.



Free ATP, isolated as a glass (by treatment of the Ba salt with H_2SO_4 and treating the concd aq soln with acetone). $[\alpha]_D^{22}$ −26.7° (c = 3.095). a_M(molar absorbancy): 15.4 × 10^3 at 259 nm and pH 7.0. Freely sol in water. A 1% aq soln has a pH of about 2 and is stable at 0° for several hrs. ATP is a tetrabasic acid and after hydrolytic cleavage it is hexabasic. It is usually pptd as the dibarium salt with 4 or 6 mols of water of crystn which can be removed by prolonged

bill of costs seeking almost $28,000.00 from plaintiff Fred Sanford ("Sanford"). Presently before the Court is Sanford's motion to review the bill of costs. For the reasons set forth below, Sanford's motion is granted, and it is ordered that the parties shall bear their own costs.

CBS contends that its request for costs is governed by Fed.R.Civ.P. 54(d), which provides in relevant part that "[e]xcept when express provision therefor is made either in a statute of the United States or in these rules, costs shall be allowed as of course to the prevailing party unless the court otherwise directs. . . ." The Seventh Circuit has held that this language creates a presumption in favor of awarding costs. *E.g., Delta Air Lines, Inc. v. Colbert,* 692 F.2d 489, 490 (7th Cir. 1982). Sanford, however, argues that Rule 54(d) is inapplicable here, and that the taxation of costs is governed solely by 17 U.S.C. § 505 of the Copyright Act. Section 505 states in pertinent part: "In any civil action under this title, the court in its discretion may allow the recovery of full costs by or against any party other than the United States or an officer thereof." This provision departs significantly from the former rule in copyright cases (under which it was mandatory to award full costs to the prevailing party), and even permits the award of costs to the losing rather than the prevailing party. *Kepner-Tregoe, Inc. v. Carabio,* 203 USPQ 124, 136 (E.D.Mich. 1979); 3 M. Nimmer, Nimmer on Copyright § 14.09 (1985).

It is clear that §505 overrides Rule 54(d) at least insofar as costs relating to Sanford's copyright claim are concerned. The usual procedure for taxing costs under Rule 54(d) was not intended to apply in any suit where there is a specific costs provision built into the controlling statute. *A. Smith Bowman Distillery, Inc. v. Schenley Distillers, Inc.,* 204 F.Supp. 374, 375 n.2, 133 USPQ 223, 224 n.2 (D.Del. 1962); *see also* Advisory Committee Note to Fed.R.Civ.P. 54(d). However, CBS asserts that Rule 54(d) still applies, to some undefined extent, to this case because Sanford's complaint also included four non-copyright counts.

[1] We disagree. From its inception, this case was essentially a copyright action. In the complaint, all the non-copyright claims were based on the same facts as Count I, the copyright claim; indeed, Sanford states that the other counts were dismissed shortly before trial "because their proofs were subsumed by the copyright claim." CBS as well has consistently focused on the copyright aspect of Sanford's suit. CBS's motion for summary judgment was directed primarily to the copyright claim and even argued that the other counts were either preempted by the Copyright Act or were essentially the same as the copyright count. CBS's reply memorandum dealt solely with the copyright claim, and the Court's opinion denying summary judgment made no distinctions between the counts, as the same set of facts applied to each of them. Moreover, CBS has not identified (and, we think, cannot identify) any cost items which relate only to the non-copyright claims and not to Count I. Thus, we find that § 505 rather than Rule 54(d) governs the taxation of any and all costs in this suit.

As noted above, § 505 vests broad discretion in the courts regarding the assessment of costs in copyright actions — no presumptions in favor of the prevailing party are created. We believe the denial of all costs is a proper exercise of our discretion under the circumstances of this case.[1] Taxation of the costs sought by CBS would impose a great hardship on Sanford and his wife, possibly driving them into bankruptcy. On the other hand, the amount of costs is immaterial to CBS a multi-billion dollar corporation. Furthermore, Sanford's case was from from meritless.[2] Although the trial took only about six days, the jury deliberated for approximately twenty-six hours. Given the close outcome of the case,[3] it would be inequitable to force Sanford into financial ruin for bringing in good faith what appeared to be a legitimate copyright claim. *Cf. Cox v. Maddux,* 285 F.Supp. 876, 883 (E.D.Ark. 1968) (in action governed by Rule 54(d), losing party should not be burdened inordinately with costs).

Accordingly, Sanford's motion to review the bill of costs is granted, and the parties shall bear their own costs. It is so ordered.

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[1] Another recent copyright case denying all costs to the prevailing party is Pacific and Southern Co., Inc. v. Duncan, 572 F.Supp. 1186, 1198, 220 USPQ 859, 864-65 (N.D.Ga. 1983), *aff'd in part and rev'd in part on other grounds,* 744 F.2d 1490, 224 USPQ 131 (11th Cir. 1984), *cert. denied,* ——— U.S. ———, 105 S.Ct. 1867 (1985). The court in that case did not explain why it declined to award costs, but the disparity in the fiscal size of the parties may well have been a factor.

[2] This is just one way in which this case is distinguishable from Williams Electronics, Inc. v. Bally Mfg. Corp., 1983 Copyright Law Decisions ¶25,582, 220 USPQ 1091 (N.D.Ill. 1983), cited by CBS.

[3] Indeed, jurors who spoke with the media reported that the debates in the jury room were heated, and the initial majority vote of the jury was in favor of the plaintiff.

Court of Appeals, Federal Circuit

Ralston Purina Company
v. Far-Mar-Co., Inc.

No. 84-1237

Decided September 19, 1985

PATENTS

1. Pleading and practice in court — Burden of proof — Validity (§53.138)

Party asserting invalidity under 35 USC 112 has initial procedural burden of going foward to establish legally sufficient prima facie case, and, if such burden is met, party relying on validity must then come forward with evidence to contrary, and court, after considering all evidence, must determine whether challenger carried burden of persuasion by clear and convincing evidence.

2. Accounting — Increased or treble damages or profits (§11.35)

Trial court's finding of willful infringement by accused infringer who was put on notice of patentee's claim by patentee's offer of license and who responded without consulting patent counsel, is not clearly erroneous, despite accused's claim that patentee withdrew offer too quickly for accused to "develop" willfulness.

Particular patents — Protein Product

No 3,940,495, Flier, Protein Product and Method for Forming Same, Claims 1-9, 14, 19, 27-31, and 33-52, invalid, Claims 10-13, 15-18, 20-26, and 32, not invalid, and Claims 10-13, 15-18, 20, 22, 23, and 32, infringed.

Appeal from District Court for the District of Kansas; Crow, J.

Action by Ralston Purina Company, against Far-Mar-Co., Inc., for patent infringement. From judgment defendant appeals. Affirmed in part and reversed in part.

Warren N. Williams and Schmidt, Johnson, Hovey & Williams, both of Kansas City, Mo. (John M. Collins, on the brief) for appellant.

Randall G. Litton and Price, Heneveld, Huizenga & Cooper, both of Grand Rapids, Mich. (Richard C. Cooper, on the brief) for appellee.

Before Baldwin and Bennett, Circuit Judges, and Miller, Senior Circuit Judge[1].

Baldwin, Circuit Judge.

The decision of the United States District Court for the District of Kansas, holding claims 1-52 of U.S. Patent No. 3,940,495 (Flier) not invalid, and holding claims 1, 2, 8-20, 22, 23, 25, and 29-33 willfully infringed, is affirmed-in-part and reversed-in-part.

Facts

The Flier invention is the first successful process, and resultant product, for directly and continuously restructuring oil seed particles, preferably soy particles, into a textured, chewable, fibrous, meat-like food product. Restructuring is accomplished by mechanically working defatted, moistened soy particles under elevated temperature and pressure, into a flowable, plastic mass which is expanded into the restructured, fibrous, meatlike food product by suddenly releasing the pressure. The original application was filed July 10, 1964. A continued-in-part application was filed December 9, 1966. A continuation application was filed January 17, 1973, from which the patent issued. Although the 1964 application is more properly called a grandparent application, it will be referred to as the parent for the purposes of this opinion.

Interference 96,355, styled *Wilding* v. *Flier* v. *Atkinson*, was declared on May 23, 1968, involving the pending patent applications of Morris Wilding (assignor to Swift and Co.), Flier (assignor to Ralston Purina Co. (Ralston)) and William T. Atkinson (assignor to Archer-Daniels-Midland Co. (ADM)). Priority was eventually awarded to Flier on August 13, 1971. The interference was appealed, but settled by a cross-licensing arrangement on April 6, 1972. The settlement agreement provided that each party would grant to any third party making a written request a nonexclusive license under the claims of any existing or future patent.

On July 9, 1973, counsel for Flier specifically advised the examiner that an ADM patent application (the Dutch publication), No. 6506477, had been published on November 22, 1965, and that it corresponded generally to the Atkinson United States patent application which had been involved in the interference. The district court found specifically that this reference was brought to the examiner's attention after discovery in June, 1973, and that it was indeed a printed publication.

Claims which correspond to claims 8, 9, and 34-52 in Flier were allowed in an office action issued April 5, 1974. Additional claims were allowed in November 1, 1974. The remaining claims were allowed June 3, 1975.

Far-Mar-Co was licensed by ADM for the product described by the patent in suit. Upon issuance of Flier, Ralston offered a license to

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[1] The Honorable Jack P. Miller assumed senior status effective June 6, 1985.

Far-Mar-Co, which was immediately declined. Ralston filed suit in district court for patent infringement approximately sixty days after Flier issued.

OPINION

The decisive issues in this case are:

1. Whether the trial court erred in deciding that Far-Mar-Co's burden of proof was to show by clear and convincing evidence that Flier was invalid.

2. Whether the trial court clearly erred in finding that the patent application of Archer Daniels Midland Company, No. 6506477 filed in the Patent Office of the Netherlands (Dutch publication) did not anticipate the claims of Flier.

3. Whether the trial court's finding that claims 10–13, 15–28, and 32 were descriptively supported by the parent application and thus entitled to the parent's 1964 filing date is clearly erroneous.

4. Whether the trial court's finding of willful infringement was clearly erroneous.

Burden of Proof

Far-Mar-Co contends that the district court erroneously imposed upon it the burden of proving insufficient disclosure in the parent application; also, that Ralston, as the party asserting adequate disclosure, should have borne the burden of demonstrating that adequate legal support exists. Ralston, on the other hand, argues that the district court correctly placed the burden of overcoming the presumption of validity by demonstrating insufficiency of disclosure of Far-Mar-Co, and found that Far-Mar-Co had not shown by clear and convincing evidence that it had met that burden.

[1] Far-Mar-Co incorrectly treats the burden of establishing a *prima facie* case of insufficiency of disclosure as if it bears no relationship to the burden of overcoming the presumption of validity accorded a patent under 35 U.S.C. § 282. A patent is presumed valid, and the burden of persuasion to the contrary is and remains on the party asserting invalidity. *W. L. Gore & Assoc., Inc.* v. *Garlock, Inc.*, 721 F.2d 1540, 1553, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 105 S.Ct. 172 (1984). In addition, the party asserting invalidity also bears the initial procedural burden of going forward to establish a legally sufficient *prima facie* case of invalidity. If this burden is met, the party relying on validity is then obligated to come forward with evidence to the contrary. Before rendering its judgment, the court must determine whether "*all* of the evidence establishes that the validity challenger so carried his burden as to have persuaded the decisionmaker that the patent can no longer be accepted as valid." *Lear Siegler, Inc.* v. *Aeroquip Corp.*, 733 F.2d 881, 885, 221 USPQ 1025, 1028 (Fed. Cir. 1984). A party asserting invalidity based on 35 U.S.C. § 112 bears no less a burden and no fewer responsibilities than any other patent challenger. Far-Mar-Co's burden of proof before the district court was to show by clear and convincing evidence that Flier was invalid. *See, e.g., Pennwalt Corp.* v. *Akzona, Inc.*, 740 F.2d 1573, 1578, 222 USPQ 833, 836 (Fed. Cir. 1984). The district court recognized and enunciated these rules. Accordingly, we hold that it did not place an impermissible burden upon Far-Mar-Co.[2] The question of whether disclosure satisfies the written description requirement of § 112 is based on questions of fact. *See In re Wilder*, 736 F.2d 1516, 1520, 222 USPQ 369, 372 (Fed. Cir. 1984), *cert. denied*, 105 S.Ct. 1173 (1985). Far-Mar-Co thus bears the burden of demonstrating that the court erred in its application of the law to the facts, *See Bose Corp.* v. *Consumers Union*, 466 U.S. 485, 104 S.Ct. 1949 (1984), or that its findings of fact were clearly erroneous.

[2] Far-Mar-Co's reliance on *Wagoner* v. *Barger*, 463 F.2d 1377, 175 USPQ 85 (CCPA 1972), explains the vehemency with which it contends that the district court erred in assigning the burden of proof. *Wagoner* involved an interference in which the senior party owned the patent whose claims were copied to provoke the interference. With respect to the burden of proof on the issue of inherency, the court stated that "[c]learly, the burden of proving that language contained in the claims of the later application [which, in this case, were allowed to issue] is on the party asserting the equivalency. . . and the burden is a heavy one." 463 F.2d at 1380, 175 USPQ at 86–87. This distinguishing feature between *Wagoner* and the present case is that *Wagoner* was an *interference*. Although the *test* for the adequacy of disclosure is the same, whether or not the proceedings are *ex parte* or *inter partes* before the Patent and Trademark Office, or before a district court, the burdens are allocated somewhat differently in each, due to their distinctive characteristics. The objective of an interference, unlike that in a district court when invalidity is alleged, is to determine priority of invention. After being accorded senior or junior party status, with the concomitant procedural benefits (senior party) or burdens (junior party), *each* party is responsible for establishing its case for sufficiency of disclosure in a prior application if it attempts to antedate a reference under § § 112 and 120 or 119. Hence, the rule enunciated in *Wagoner*, 463 F.2d at 1380.

Anticipation

Anticipation is a factual determination, reviewable under the "clearly erroneous" standard. *Lindemann Maschinenfabrik GMBH* v. *American Hoist & Derrick Co.*, 730 F.2d 1452, 1458, 221 USPQ 481, 485 (Fed. Cir. 1984). A finding is "clearly erroneous" when although there is evidence to support it, the reviewing court on the entire evidence is left with the definite and firm conviction that a mistake has been committed. *United States* v. *U.S. Gypsum Co.*, 333 U.S. 364, 395, 76 USPQ 430, 444 (1948); *SSIH Equipment S.A.* v. *USITC*, 718 F.2d 365, 381, 218 USPQ 678, 692 (Fed. Cir. 1983). Anticipation requires that "all limitations of the claim are found in the reference, or 'fully met' by it." *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760, 772, 218 USPQ 781, 789 (Fed. Cir. 1983).

Ralston contends, and the trial court found, that the Dutch publication was deficient because it failed to disclose (1) the importance of a "minor amount of fat" present during extrusion; (2) a definition for the word "plexilamellar" which was used to describe the product; and (3) the desirability of "separate zones of confinement or orifices" in the machinery used to mix and extrude the product. These findings are clearly erroneous.

The first finding is clearly erroneous because the Dutch publication specifies the same starting material claimed in Flier, and the trade defines the starting material as having low levels of fat. The publication is therefore not deficient as to this element of Flier's claims.

The second finding, that the Dutch publication does not define "plexilamellar" is unsupportable in view of the following passage from page 2 of that publication:

> The protein extrudate obtained according to the above mentioned method is a rough, resilient, dry to slightly moist to the touch, open celled foamy mass made up of interlaced interconnected strips of varying width and thickness which may appear fibrous or skin-like. The majority of the cells formed by this *plexilamellar* protein structure are . . . . [Emphasis added.]

The third finding is similar to the first, and fails for a similar reason. The Dutch publication discloses the use of a standard extruder which, at the time, came equipped with the structure specified in the Flier patent. The publication is therefore not deficient as to this element of Flier's claims.

As a result of our disposition of this issue, only those claims entitled to the effective filing date, July 10, 1964, of the parent application remain in issue. The trial court held and Ralston does not contest, that claims 1–9, 14, 29–31, and 33–52 were entitled only to the effective filing date of the 1966 application. Thus, we hold these claims to be invalid for having been described in a printed publication before the invention thereof by the applicant for patent. 35 U.S.C. § 102(a).

Description Requirement

The trial court held that claims 10–13, 15–28, and 32 of Flier are entitled to the effective filing date of the 1964 parent application because the parent application complies with the written description requirement of 35 U.S.C. § 112, first paragraph, which is incorporated in 35 U.S.C. § 120. Whether the description requirement is met is a question of fact reviewable under the clearly erroneous standard. *In re Wilder*, 736 F.2d 1516, 1520, 222 USPQ 369, 372 (Fed. Cir. 1984), *cert. denied*, 105 S.Ct. 1173 (1985). The trial court properly recognized that the test for sufficiency of support in a parent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter." *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983). Precisely how close the original description must come to comply with the description requirement of 35 U.S.C. § 112 must be determined on a case-by-case basis. *In re Wilder*, 736 F.2d 1516, 1520, 222 USPQ 369, 372 (Fed. Cir. 1984).

Far-Mar-Co cites several range cases to support its argument that ranges found in the applicant's claim language must correspond exactly to ranges disclosed in the parent. These cases are not in point. The facts in these cases precluded a determination that one skilled in the art could derive the claim limitations from the parent, due to a number of different factors, e.g., the unpredictable nature of the art, *In re Sichert*, 566 F.2d 1154, 196 USPQ 209 (CCPA 1977); failure to distinguish one process from another, *In re MacLean*, 454 F.2d 756, 172 USPQ 494 (CCPA 1972); the addition of a critical limitation, *In re Blaser*, 556 F.2d 534, 194 USPQ 122 (CCPA 1977); failure to define a critical term, *In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971); and use of a list that did not contain the claimed substance. *In re Ahlbrecht*, 435 F.2d 908, 168 USPQ 293 (CCPA 1971). In addition, a predecessor to this court has held "that a claim may be broader than the specific embodiment disclosed in a specification is in itself of no moment." *In re Rasmussen*, 650 F.2d 1212, 1215, 211 USPQ 323, 326 (CCPA 1981). Far-Mar-Co argues that the claims

remaining in issue contain new matter at least with respect to the protein content of the starting material, total and added moisture, temperature ranges, and the situs of fiber formation. Far-Mar-Co contends that although the 1964 parent application would enable one skilled in the art to practice the invention claimed, it does not meet the description requirement under 35 U.S.C. § 112.

With respect to protein content, Far-Mar-Co argues that the claim language "protein content of at least about that of solvent extracted soybean meal" is not supported by the language of the parent application, which speaks of "soybean meal having a low fat and high protein content." The parent application also states that "[s]uch 50% protein soybean meal is well known and frequently is a by-product of the process of oil extraction from soybeans. Such meal is preferably solvent extracted to decrease the fat content thereof to the range mentioned above." Further, "[s]oybean meal having a protein content of approximately 50% is the preferred meal component for use in the present invention. When, however, the meal has a protein content of substantially less than 50%, it may be mixed with a high protein component which will increase the protein content of the combination to the preferred 50%."

The trial court found that the parent disclosure does support the claim language, based on the 1964 disclosure and on consideration of the knowledge possessed by those skilled in the art of extrusion of both farinaceous and proteinaceous vegetable materials in 1964. The trial court found that soybean meal of 44%, 50%, 70%, and 90% protein were standard, available commodities in 1964. The trial court also found that the parent, which disclosed a "high protein content" and a preferred lower level but no upper limit, and indicated that protein content could be adjusted, reasonably conveyed adjustment of the protein content of soybean meal to levels above 50%. Having considered Far-Mar-Co's arguments, we conclude that the court did not clearly err in determining that the parent's disclosure adequately supports the protein content of the claims in issue.

With respect to temperature, Far-Mar-Co argues that the claim limitation "in excess of 212° F" and "substantially above 212° F" are not supported by the parent application. The trial court found that experts from both parties were in substantial agreement that the parent application sets the critical lower limit for temperature at 212° F and supports this limit in the patent claims. The trial court considered evidence of what the skilled artisan would appreciate about the sources of heat in the process, both steam heat and pressure brought to bear on the mixture, as well as the limitations of the equipment disclosed. The trial court also noted that Far-Mar-Co's expert agreed that the claim language calling for the temperature "being increased substantially" found support in the parent application. On the basis of this record, it was not clear error for the court to find sufficient disclosure in the parent application for the above-mentioned limitations.

Far-Mar-Co argues that the trial court clearly erred in finding support in the parent for the moisture content limitations. The trial court considered (1) evidence that the purpose of moisture in the mix was to make the material flow through the extruder; (2) the physical characteristics of mixtures with varying levels of water; (3) the type of test and degree of accuracy in testing for moisture level; and (4) the approximate amount of moisture known by those skilled in the art to be contained in soybean meal. Based on this evidence and the formulations disclosed in the parent application, the court allowed both parties to calculate approximate upper and lower moisture limits supportable by the parent application. It found inadequate descriptive support in the parent application for the moisture limitations of "at least about 20%" and of those claims calling for a total moisture content "between about 20% and 40% by weight," and the parties do not contest these findings. The court found adequate support for moisture levels of "at least about 25% by weight," "at least 25% by weight," and "in the range of 20-30% of the resulting mixture." The trial court noted that claims simply calling for sufficient water to permit the resulting mixture to be passed through an extruder or calling for approximately 25% of the mixture were not challenged. The trial court's rationale for striking down the claims with endpoints of 20% and 40% was that these limits could not be justified solely by the so-called ball test for moisture content. Those claims would convey new information to one skilled in the art. The open-ended claims, however, would be limited by what a person skilled in the art would understand to be workable. After careful consideration of Far-Mar-Co's arguments, we conclude that the court did not clearly err in determining that the parent's disclosure adequately supported the water ranges of "at least about 25% by weight," and "at least 25% by weight." The court, however, did clearly err in finding support in the parent for the limitation: "in the range of 20%-30% of the resulting mixture" contained in claims 19, 27, and 28. We hold these claims are entitled only to the effective filing date of the 1966 application and are therefore invalid for having been anticipated by the Dutch publication.

Far-Mar-Co's argument that the parent application requires fiber formation inside the extruder is adequately disposed of by the trial court's opinion.

In sum we conclude that claims 10-13, 15-18, 20-26, and 32 of the Flier patent are entitled to the effective filing date of the 1964 parent application because the parent application adequately supports those claims for purposes of 35 U.S.C. § 120.

Willful Infringement

A finding or willful infringement is a question of fact and is not reversible upon appeal unless shown to be clearly erroneous. *Underwater Devices, Inc. v. Morrision-Knudsen Co.*, 717 F.2d 1380, 1389, 219 USPQ 569, 576 (Fed. Cir. 1983). Far-Mar-Co has failed to persuade us that the district court's finding is clearly erroneous.

[2] The trial court found willful infringement based on Far-Mar-Co's "conduct after issuance of the patent, particularly the decision to respond to plaintiff's offer of a license without consulting patent counsel." When a potential infringer has actual notice of another's patent rights, he has an affirmative duty to exercise due care to determine whether or not he is infringing. *Underwater Devices, Inc.* v. *Morrison-Knudsen Co.*, 717 F.2d at 1389-90, 219 USPQ at 576. Such an affirmative duty usually includes, inter alia, the duty to seek and obtain competent legal advice from counsel before the initiation of any possible infringing activity. *Id. See also, King Instrument Corp.* v. *Otari Corp.*, 767 F.2d 853, 867, 226 USPQ 402, 412 (Slip op. pp. 31-32, June 26, 1985). The offering of a license is actual notice. *Leinhoff* v. *Louis Milona & Sons, Inc.*, 726 F.2d 734, 743, 220 USPQ 845, 851 (Fed. Cir. 1984).

Far-Mar-Co's argument that it did not infringe willfully because Ralston withdrew its offer too quickly is unpersuasive. There is evidence of record that Far-Mar-Co has known of its potential infringement liability to Ralston since 1970. At that time, Ralston was involved in an interference proceeding with other parties, one of whom was Far-Mar-Co's licensor. Far-Mar-Co received notice that Ralston won the interference in April, 1972. After the Ralston patent issued in 1976, Far-Mar-Co rejected a license offer without even consulting its own in-house patent counsel. Far-Mar-Co cites no precedent for a decision that an infringer must be allowed a certain amount of time to "develop" willfulness, and we will not supply it. On the basis of this record, we cannot say the court clearly erred in its finding.

Accordingly, we agree with the district court's decision that claims 10-13, 15-18, 20-26, and 32 of the Flier patent have not been shown to be invalid. We *affirm* the holding of infringement of claims 10-13, 15-18, 20, 22, 23, and 32. We hold claims 1-9, 14, 19, 27-31, and 33-52 to be invalid. Finally, we *affirm* the finding of willful infringement.

AFFIRMED-IN-PART AND REVERSED-IN-PART.

Miller, Senior Circuit Judge, dissenting in part and concurring in part.

I cannot agree with the section in the majority opinion concerning the "written description" requirements of 35 U.S.C. § 112, first paragraph with respect to claims 10-13, 15-28, and 32.

It is necessary that Flier be entitled to its grandparent application's filing date under 35 U.S.C. § 120 if it is to avoid the invalidating effect of the ADM anticipating reference under 35 U.S.C. § 102(a). To be entitled to the benefit of the date of a previously filed copending application under section 120, such application must contain a written description of the invention claimed, and of the manner and process of making and using it, as set forth in the later application to comply with the first paragraph of section 112.

The invention claimed in the later application does not have to be described in the prior application *in ipsis verbis* in order to satisfy the description requirement of section 112. *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972); *see Case v. CPC International, Inc.*, 730 F.2d 745, 751, 221 USPQ 196, 201 (Fed. Cir.), *cert. denied*, 105 S.Ct. 223, 224 USPQ 736 (1984). However, claims with no explicit disclosure must find inherent support in the prior application, *Pingree v. Hull*, 518 F.2d 624, 186 USPQ 248 (CCPA 1975); and one skilled in the art, *following the teaching* of the prior application must be able to produce the subject matter of the later claims. *In re Magerlein*, 346 F.2d 609, 612, 145 USPQ 683, 685 (CCPA 1965); *In re Nathan*, 328 F.2d 1005, 1008-09, 140 USPQ 601, 604 (CCPA 1964). Thus, the test for determining whether the disclosure complies with the written description of the invention requirement is whether it would have reasonably conveyed to one of ordinary skill that the inventor invented the later-claimed subject matter. *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983). The "legal' equivalent of the claim language is

thus the "necessary and only reasonable construction" to be given the disclosure in the parent application by one skilled in the art. *In re Filstrup*, 251 F.2d 850, 853, 116 USPQ 440, 442 (CCPA 1958). The result claimed must "inevitably occur." *See Kooi v. DeWitt*, 546 F.2d 403, 409, 192 USPQ 268, 273 (CCPA 1976); *Pingree*, 518 F.2d at 627, 186 USPQ at 251.

Section 112 does not refer to a mere "support" standard. In *In re Smith*, 458 F.2d 1389, 1394, 173 USPQ 679, 683 (CCPA 1972), the court stated that "[t]he recent cases suggests [*sic*] a more stringent requirement for a description of the claimed invention than may have been previously applied in cases wherein the issue was framed in terms of 'support' for claimed subject matter." The original disclosure may not be relied upon unless it "constitute[s] a full, clear, concise and exact description . . . of the invention claimed" in the patent to one of ordinary skill. *In re Wertheim*, 646 F.2d 527, 538-39, 209 USPQ 554, 565 (CCPA 1981) ("*Wertheim II*").

The test of adequacy of disclosure is neither anticipation (*e.g., In re Scheiber*, 587 F.2d 59, 199 USPQ 782 (CCPA 1978)) nor obviousness (*See, e.g., In re Piasecki*, 745 F.2d 1468, 1473, 223 USPQ 785, 789 (Fed. Cir. 1984)). Thus, it is not proper under section 112 to require that a person of ordinary skill determine by "extrapolation, interpolation and assumptions" (*Ex parte Eggleston*, 159 USPQ 692, 693 (PTO Bd. App. 1967)) that disclosure in the prior application would achieve a product possessing characteristics of, or operating within the ranges of numerical values set forth in, the later claimed subject matter. *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976) ("*Wertheim I*"), *later appealed*, 646 F.2d 527, 209 USPQ 554 (CCPA 1981).

To carry its burden of demonstrating insufficient disclosure, Far-Mar-Co must show that the grandparent application would not have taught persons skilled in the art that the ranges of each item claimed in the patent claims were Flier's invention.[1]

With respect to protein content of the soybean meal, Far-Mar-Co asserts that the requirements in the Flier patent claims for "vegetable material having a protein content of at least about that of solvent-extracted soybean meal" (claims 15-17 and 32) and for "solvent-extracted soybean material having a protein content at least about that of solvent-extracted soybean meal" (claims 18-26) are not inherent in the grandparent specification, since each claim limitation is an open-ended range of up to 100% protein.

The grandparent contains the following references to soybean meal protein:

> It has been found that Soybean meal having a low fat and *high protein content* may be treated to form the desirable products of the present invention. Preferably the soybean meal has a fat content as low as 0.5% and a *protein content of approximately 50%*. Such *50% protein soybean meal* is well known and frequently is a by-product of the process of oil extraction from soybeans. . . .
>
> . . . .
>
> *Example 1* As a specific example, 17 pounds of 50% *soybean meal having a protein content of 50%*, a fat content of. . . .
>
> *Example 2*
>
> . . . .
>
> *Soybean meal having a protein content of approximately 50%* is the preferred meal component for use in practicing the present invention. *When, however, the meal has a protein content of substantially less than 50%*, it may be mixed with a high protein component which will *increase the protein content of the combination to the preferred 50%*.

(Emphasis supplied.) The district court noted that most of the references to protein concentrations in the grandparent application emphasize the importance of "approximately" 50% protein soybean meal. It also found that in Example 2 of the grandparent indicates that soybean meal lacking sufficient protein concentration may be altered to produce the preferred percentage.

The court also referred to the Soybean Blue Book for 1964 and the Yearbook and Trading Rules for 1964-1965, in which it found reflected the knowledge in the art that the protein content of 50% solvent-extracted soybean meal was "minimum 50%" and that 44% soybean meal was also available. The court also noted that soybean protein concentrate and soybean protein isolate (not soybean meal as the majority opinion suggests), with protein concentrations over 50%, were "well known" in the art in 1964.

I am persuaded that the district court erred when it found from these references (in combination with the grandparent specification disclosure of "about 50% and "approximately 50%" protein) that "[i]t is doubtful that a person skilled in the art would . . . have construed from the parents *[sic]* disclosure a maximum protein limit of about 50%." (Finding 139.) By assuming that those of ordinary skill in 1964 would have had additional reference materials for use in creating limitations in the grandparent specification, the court erroneously applied an obviousness analysis, transgressing this court's declaration in Piasecki, 745 F.2d at 1473, 223 USPQ at 789, and *In re Shetty*, 566 F.2d 81, 86, 195 USPQ 753, 756 (CCPA 1977), *reh'g denied* (Jan. 19, 1978), that the specification itself must be the source of its interpretation (with respect to scope) for one of ordinary skill. *In re Ruschig*, 379 F.2d 990, 995-96, 154 USPQ 118, 123 (CCPA 1967).

The district court also found that "[a]djustment of the protein content of soybean meal to a level above 50% is reasonably conveyed where the disclosure set forth a requirement of a 'high protein content,' disclosed the preferred level but no upper limit. . . ." By taking this language out of context, the court erred in its interpretation of the specification. The language should have been read *in pari materia* with the sentence that follows it, which clearly indicates that Flier equated "high protein content" with "approximately 50%." I disagree with the gloss imposed on the language of the grandparent application by the majority opinion. The *patent* claim language is not the only *"necessary and reasonable"* construction of the language in the grandparent application *(In re Filstrup*, 251 F.2d at 853, 116 USPQ at 442), and a "level above 50%" is not the "inevitable" interpretation of "about," "approximately," or even "preferably" 50%. *Cf. Kropa v. Robie*, 187 F.2d 150, 154-55, 88 USPQ 478, 483 (CCPA 1951).

The district court conceded that the "open-ended range of from about 50% to 100% is "in part predicated on the assumption that solvent-extracted soybean meal contains about 50% protein." If this means, as the majority asserts, that the district court found that the grandparent application discloses a "preferred lower limit," such finding is clearly erroneous. The grandparent application does state that 50% protein meal was the preferred concentration. However, in view of the language in the grandparent application expressing Flier's knowledge that "substantially less than 50% protein was a class of protein concentrations known to him at that time (which concentrations were readily raised to the preferred 50%), I cannot agree that the grandparent taught 50% protein concentration as a "preferred lower limit." Rather, if "preferred" is to be interpreted as a limitation, the more reasonable construction in this case would be as an upper limit. I conclude that the district court erred in finding that the Flier patent claims containing limitations on protein content of soybean meal were sufficiently disclosed in the 1964 grandparent application.

With respect to moisture content, Far-Mar-Co contends that the district court erred in concluding that the Flier claims limitations were sufficiently disclosed in the grandparent application. The 1964 grandparent recites, in the examples, 17 pounds of soybean meal "mixed with 2600 cc. of water" or 2850 cc. (It is undisputed that 2600 and 2850 cc. are 25 and 27% by weight, respectively, of the mixtures recited in the examples.) The claims limitations refer to "at least about 25% by weight" (claims 10-13) and "at least 25% by weight" (claims 15-17, 32).

These findings by the district court suffer from the same infirmities as do those with respect to protein concentration. Although written disclosure cases must be determined on a case-by-case basis (*e.g., In re Driscoll*, 562 F.2d 1245, 1250, 195 USPQ 434, 438 (CCPA 1977)) *Wertheim I, supra*, opposes extending, without limitation, the range of the only examples stated in the prior application in a situation similar to the present claims 10-13, 15-17, and 32. *See In re Ahlbrecht*, 435 F.2d 908, 168 USPQ (CCPA 1971); *Smith*, 458 F.2d at 1394-95, 173 USPQ at 683 (disclosure of genus and one species not sufficient description of intermediate subgenus). Certainly, genera and subgenera ranges which substantially deviate from the two species disclosed in the grandparent are not sufficiently described when there is no suggestion to those skilled in the art that such ranges of moisture are embraced by the original invention.

In reaching its conclusions, the district court relied on (1) "the practice" at Ralston in 1964, (2) "squeeze test," and (3) knowledge of those skilled in the art of the moisture content of soybean meal. It should be pointed out that "the practice" at Ralston in 1964 does not even appear to be within the knowledge of one of ordinary skill, and neither a "squeeze test" nor the importance of the moisture content of soybean meal is suggested in the grandparent application. *Cf. In re Salmon*, 705 F.2d 1579, 1581, 217 USPQ 981, 983 (Fed. Cir. 1983); *Wertheim I*, 541 F.2d at 267-68, 191 USPQ at 101. I am persuaded that the district court's findings on moisture content limitations are clearly erroneous.

Far-Mar-Co also contests the findings of the district court on the issue of the range of processing temperatures. The 1964 application recites a range of 212-360° F in one example and states elsewhere that the mixture "must be subjected to heat . . . during the extrusion process." The Flier patent claims recite "in excess of 212° F" (claim 10) and "substantially above 212° F" (claims 11-13, 15-16, 28, and 32). These findings of the court are subject to the same criticism as are those

[1] The sufficiency of Far-Mar-Co's *prima facie* case is not at issue, since the district court evidently considered all of the evidence produced by both parties.

relating to moisture content. The reasoning in *Wertheim I, Ahlbrecht,* and *Smith* applies to the limitations in claims 10, 15–16, 28, and 32. "[I]nto the range of 212–310° F" (claim 27) requires further discussion, because this range is totally within the range explicitly disclosed in the grandparent.

The court in *Wertheim II* held that the disclosure in the parent of 25% to 60% solids content, without more, did not satisfy the description requirement of the later claimed 35% to 60% solids concentration, because the claimed range was a significant restriction on the invention. 646 F.2d at 538, 209 USPQ at 565. Analogous is claim 27, in which Ralston attempts to rely upon the grandparent's disclosure of 212–380° F. Although it is likely, as the district court states, that "the skilled artisan would observe a practical upper limit of avoiding burning or scorching material passing through the extruder" (Finding 151), the ADM reference indicates that extrusion temperatures of 450°F were feasible in 1965. Without any suggestion in the grandparent that temperatures above 310° F would damage the product, there is no "inevitable" or "necessary and only reasonable construction" of the grandparent's disclosure as having the upper limit of 310° F in claim 27. *Cf. In re Salmon,* 705 F.2d at 1581, 217 USPQ at 983.

Finally, on the issue of situs of fiber formation (claims 10–13, 15–28, and 32), I concur with the majority opinion that Far-Mar-Co has not demonstrated that the district court clearly erred. Although Flier did not know in 1964 where fiber formation occurred, I am persuaded that one of ordinary skill in the art would have been taught by the grandparent disclosure how the invention was practiced. *See Spero v. Ringold,* 377 F.2d 652, 656, 153 USPQ 726, 728–29 (CCPA 1967); *In re Magerlein,* 346 F.2d at 611–12, 145 USPQ at 685. The invention "may well [have been] disclosed without positive identification." *Petisi v. Rennhard,* 363 F.2d 903, 907, 150 USPQ 669, 672 (CCPA 1966); *see Foss v. Oglesby,* 127 F.2d 312, 317, 53 USPQ 356, 361 (CCPA 1942).

In view of the foregoing, Ralston cannot rely on Flier's 1964 grandparent application for priority under sections 112 and 120 for claims 10–13, 15–28, and 32. Thus, these claims are rendered invalid by the ADM anticipating reference.

I do not join the majority on the issue of willfulness because it is rendered moot in light of my dissent.

District Court, E. D. Pennsylvania

Associated Film Distribution Corporation, et al.
v. Thornburgh, et al.

No. 80-1179

Decided August 5, 1985

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1. In general (§24.01)

Pennsylvania Feature Motion Picture Fair Business Practice Law, which regulates licensing of feature films by prohibiting "blind bidding," by prohibiting guarantees and advances for feature film exhibition, and by prohibiting film distributors from granting exhibitors exclusive "first runs" of films for more than 42 days, does not violate First Amendment, nor is it preempted by Copyright Act.

Action by Associated Film Distribution Corporation et al., against The Honorable Dick Thornburgh, Governor of Pennsylvania, Attorney General, Commonwealth of Pennsylvania, Budco Quality Theatres, Inc., and Fox Theatres Management Corporation et al., for declaration of constitutionality of Pennsylvania Feature Motion Picture Fair Business Practices Act. Act held to be constitutional.

See also 214 USPQ 742 and 216 USPQ 184.

Carole E. Handler, Dennis R. Suplee, David G. Battis, and Schnader, Harrison, Segal & Lewis, all of Philadelphia, Pa., and Barbara Kacir, and Jones Day Reavis & Pogue, both of Cleveland, Ohio, for plaintiffs.

Gregory R. Neuhauser, for state defendants.

Richard M. Squire, Alan M. Lerner, Peter M. Fishbein, and Karen E. Katzman, all of New York, N.Y., for defendant Fox Theatres Management Corporation.

H. Donald Busch, Karin Anita von Dreusche, and Busch & Schramm, all of Bala Cynwyd, Pa., for defendant Budco Quality Theatres, Inc.

Katz, District Judge.

FINDINGS OF FACT AND CONCLUSIONS OF LAW

I uphold the constitutionality of the Pennsylvania Feature Motion Picture Fair Business Practices Law.[1] That Act regulates certain trade practices in the licensing relationship between those who distribute films and those who exhibit them. The distributors contend that the Act violates their rights to freedom of speech, offends the right of Congress to regulate interstate commerce, is preempted by federal copyright legislation, and is contrary to the Pennsylvania Constitution's prohibition of "special laws."[2]

I. *Background: Mutual Dependency*

The relationship between exhibitors and distributors is one of mutual dependence. Distributors need theatres in which to play their films; exhibitors need films to play in their theatres. This symbiotic relationship has given rise to a long history of sharp dealing. *See United States v. Paramount Pictures,* 334 U.S. 131, 77 USPQ 243 (1948).

The plaintiffs in this action are the major distributors of motion pictures in the United States. They distribute most films. Those which they do not distribute are released by foreign companies or small independents.

Although there are national theatre chains, most theatres in Pennsylvania are owned by local chains or by independent exhibitors. The exhibition business in some areas of the Commonwealth is competitive, while in other areas a chain or an individual owns all the theatres. If a distributor wishes to play a film in a certain area, it must license that film to an exhibitor in that area. Not all theatres are alike. Factors such as seating capacity, location, and parking are important in what is a prime theatre.

All exhibitors depend on the product available from distributors. Theatre owners need new pictures on their screens. Exhibitors fear the mythical screen without a picture; distributors fear the mythical film without a theatre.

During the peak seasons, Christmas, Easter and summer, there is usually ample product for exhibitors. At other times, however, distributors release fewer films. This is a problem for both small exhibitors operating one theatre and for the giants of the exhibition field who own all the screens in an area. The more screens an exhibitor owns, the more devouring its need for film product. While a theatre owner can subsist on the average film, the eternal hope is to garner a blockbuster.

Distributors license films by two methods, bidding and negotiation. The Pennsylvania Act defines a bid as

> [a] written or oral proposal by an exhibitor to a distributor, which proposal is in response to an invitation to bid or negotiate and states the terms under which the exhibitor will agree to exhibit a feature motion picture.
>
> 73 P.S. § 203–3

In practice, distributors use bidding in areas where competition among exhibitors exists. Although bidding may take place over the telephone, normally the distributor sends exhibitors in the area an "invitation to bid" which contains a brief description of the film, the time at which it will be available and the suggested licensing terms. The exhibitor submits a bid which will include not only financial terms, but also minimum length of run and any requested "clearances" over other theatres in the area. A clearance is an assurance that another theatre will not obtain the same film. The distributor, taking into account both the quality of the theatre and the terms of the bid, presumably selects the most favorable bid. The distributor can reject all bids. Before the Pennsylvania Act, the distributor could negotiate with individual theatres after rejecting all bids. Traditionally, terms on licenses procured by bidding are "firm"; the distributor will not be expected to reduce the agreed terms if the picture is unsuccessful.

Distributors normally use negotiation in areas where there is little or no competition among exhibitors. Under negotiation, a representative of the distributor contracts a specific exhibitor and, without soliciting other offers, attempts to work out a licensing arrangement. Traditionally, terms under negotiated licenses are not firm. If a picture bombs, the distributor may renegotiate the terms downward.

Another method of licensing is "competitive negotiation." Competitive negotiation is oral bidding. The distributor contacts the exhibitor and indicates that he is soliciting offers from more than one theatre. As with bidding, the industry practice is that terms licensed under competitive negotiation are considered firm.

---

[1] 73 P.S. § § 203–1 through 203–11.

[2] The history of this case includes a grant of summary judgment for the plaintiffs on the grounds that the Act violated the First and Fourteenth Amendments and the Supremacy Clause of the United States Constitution. *See Associated Film Distributors, et al. v. Thornburgh,* 520 F.Supp. 971, 214 USPQ 742 (E.D.Pa. 1981) (hereinafter *AFD* I). The Court of Appeals reversed the order. 683 F.2d 808, 216 USPQ 184 (3d Cir. 1982) (hereinafter *AFD* II).

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URKUNDE

Es wird hiermit bescheinigt, daß für die in der beigefügten Patentschrift beschriebene Erfindung ein europäisches Patent für die in der Patentschrift bezeichneten Vertragsstaaten erteilt worden ist.

CERTIFICATE

It is hereby certified that a European patent has been granted in respect of the invention described in the annexed patent specification for the Contracting States designated in the specification.

CERTIFICAT

Il est certifié qu'un brevet européen a été délivré pour l'invention décrite dans le fascicule de brevet ci-joint, pour les Etats contractants désignés dans le fascicule de brevet.

Europäisches Patent Nr.: 0063879
European patent No.:
Brevet européen n°:

Patentinhaber:
Proprietor of the patent:
Titulaire du brevet:

YALE UNIVERSITY
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New Haven, CT 06520/US

München, den 22.11.89

J. Infante

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APPLICANT: DAVID C. WARD ET AL.

TITLE: MODIFIED NUCLEOTIDES AND METHODS OF PREPARING AND USING SAME

ENCLOSED ARE:

- ☐ Application
- ☐ Declaration
- ☐ Drawings
- ☐ Assignment
- ☐ Response to OA
- ☐ Amendment
- ☐ Issue Fee
- ☒ Other (see below)

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FILED IN PERSON: APRIL 22, 1993

Serial No. 07/886,660

Docket No. ENZ-1 (DIV. I)

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APPLICANT: DAVID C. WARD ET AL.

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Serial No. 07/886,660

Docket No. ENZ-1 (DIV. I)

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FARMINGDALE, N.Y. 11735

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☐ This notice is issued in view of applicant's communication filed _____

SERIES CODE/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
07/532,704	06/04/90	031	ROLLINS, J 1803	09/16/92

First Named Applicant: ENGELHARDT, DEAN

TITLE OF INVENTION: BASE MOIETY-LABELED DETECTABLE NUCLEOTIDE (AS AMENDED)

BOG	ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1	ENZ-5DIV.4	536-027.000	633	UTILITY	NO	$1130.00	12/16/92

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SERIES CODE/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
07/130,878	12/09/87	142	MARSCHEL, A.	12/03/93

First Named Applicant: WARD, DAVID C.

TITLE OF INVENTION: METHODS OF USING LABELED NUCLEOTIDES (AS AMENDED)

	ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1	CNZ-100CNT2	435-006.000	320	UTILITY	NO	$1170.00	03/03/94

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